The physiology of sporophore initiation in the cultivated mushroom Agarious bisporus (Lange), Singer.

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The Physiology of Sporophore Initiation

in the Cultivated Mushroom

Agaricus bisporus (Lange), Singer

submitted by P.E. Long

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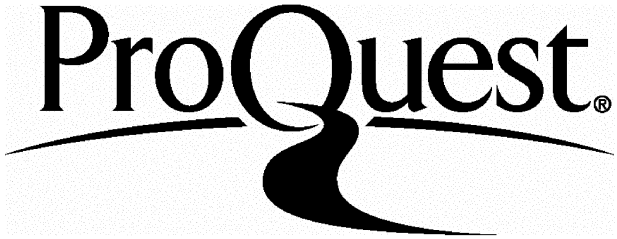

D E D I C A T I O N

To my wife

ACKNOWLEDGEMENTS

I wish to thank Professor L. Broadbent for provision of research facilities and Dr. L. Jacobs for his advice, encouragement and helpful criticism throughout this study. I must also express my gratitude to Wrington Vale Nurseries Ltd. for provision of compost and casing materials; to White Queen Ltd. for mushroom cultures and to Mr. D. Bennett for advice and assistance with the photography.

SUMMARY

The relationship between sporophore initiation in *Agaricus bisporus* and the function of the casing layer has been investigated. Initiation was found to be controlled by carbon dioxide and the activity of micro-organisms. No evidence was discovered for the involvement of other volatile factors.

Vegetative growth into compost and casing showed an absolute requirement for CO_2. Sporophore initiation and early development of carpophores also required exogenous CO_2. Optimal CO_2 levels for initiation lay between 340 and 1000 p.p.m. Above these CO_2 concentrations vegetative growth was enhanced with a concommitant decline in the numbers of fruit bodies produced.

Although no specific active micro-organisms could be isolated earlier reports of microbial involvement were confirmed. It was not possible to induce carpogenesis in sterile casing materials other than gas adsorbent charcoal. It is suggested that micro-organisms could function in initiation through irreplacable removal from hyphal apices of metabolites promoting vegetative growth. These substances may be synthesised from the products of CO_2 fixation, which could account for the suppression of carpogenesis by excess CO_2.

CONTENTS

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INTRODUCTION

Cultivation of Agaricus bisporus (Lange), Singer originated in France in the seventeenth century, probably associated with the use of hot beds for melons (Treschow, 1944; Ramsbottom, 1953). Among the early accounts, summarised by Ramsbottom those of La Quintinye in 1690 and Lister in 1698 described production of mushrooms on ridged piles of horse manure covered (i.e. cased) by a layer of soil. This technique of covering composted manure with a casing layer continues to form the basis of commercial culture. Peat has superseded soil as a casing material in this country, but the latter is still widely used in the United States and to a lesser extent in Europe. The function of the casing layer is to promote sporophore formation since few fruit bodies develope on uncased compost (Schisler, 1957; Eger, 1961). The process of sporophore initiation in the cultivated mushroom has been described by Atkinson (1906) and Hein (1930). Growth of directionally orientated hyphal strands from the compost into the casing is followed by formation, at the strand tips, of the loosely tangled hyphal aggregates comprising the early primordium. Despite several investigations in the last thirty years the inducing mechanism functioning in the casing remains unknown.

Early speculation on the nature of the fruiting stimulus was largely unsupported by experimental evidence. Thus Magnus (1906); Styer (1930) and Hein (1930) associated initiation with improved aeration as hyphae neared the casing surface. In a review of mushroom culture, Lambert (1938) proposed that a critical factor could be differences in carbon dioxide pressure between compost and casing, but suggested an alternative view that the casing might

function as a nutritionally deficient layer. This latter concept agreed with the views of Klebs (1900) on control of reproduction in fungi, which were restated with reference to the cultivated mushroom by Edwards, (1949). However, Schisler (1957) successfully employed ground compost as a casing layer, apparently refuting this hypothesis. Fruiting was considerably reduced and delayed under these circumstances, a finding confirmed by Eger (1961) who noted that although washing or homogenising compost used in this way was beneficial, the yield of mushrooms was still very much below that obtained with more conventional casing materials. These observations throw doubt on Schisler's dismissal of the Klebsian hypothesis, since homogenisation or washing may have resulted in a loss of readily available nutrients. Recently, the applicability of Klebs' postulates to fungal reproduction has been re-emphasised by Morton (1967).

The first experimental approach to the problem of sporophore initiation in the cultivated mushroom was conducted by Mader (1943) who postulated that the process was controlled by unidentified volatile factors. Mushrooms were grown in sealed containers to simulate cave conditions, employing air recirculated through a variety of washes including mineral oil and alkali. On the basis of his observation that circulation of mineral oil washed air promoted sporophore initiation, Mader suggested that toxic materials inhibiting carpogenesis were being removed by this treatment and thought that these might be hydrocarbons which were being absorbed by the oil. Many of Mader's experiments were repeated by Tschierpe (1959); Tschierpe and Sinden (1964), who concluded that his findings

could be explained by uncontrolled experimental fluctuations in carbon dioxide concentrations.

Stoller (1945, 1952) performed experiments similar to those of Mader using earthenware pots sealed to bell-jars by wide rubber bands. Air was recirculated via an alkali wash to remove CO_2 and and attempt was made to compensate for respiratory oxygen uptake. Mature sporophores did not develope in the absence of carbon dioxide, but passage of the alkali washed air through casing soil permitted normal fruit body formation. These results were interpreted as indicating the existence of volatile inhibitors produced by mushroom mycelium which were inactivated in the casing layer. Metabolic CO_2 however, would have been present in Stoller's experimental system, the alkali wash merely preventing any accumulation in the bell-jars. Similar criticisms can be applied to Stoller's (1952) account of abnormal development of young mushroom primordia in carbon dioxide free air. Here Stoller conjectured that the volatile materials had an antibiotic-like activity for maintenance of vegetative growth, and suggested they were quininoid in nature because of their low volatility and apparent high oxidising capability. The latter was demonstrated by colour changes in gum quiacum or benzidine soaked filter papers suspended over pots of mycelium impregnated compost. Stoller considered that these compounds were inactivated in the casing through the existence of high redox potentials there. This concept was disproved by the successful use of acid washed sand as a casing (Schisler, 1957).

Schisler (1957) also emphasised the importance of volatiles produced by the mycelium, but suggested a hormonal function for

these materials. The casing layer was considered to be a zone in which initiation promoting substances, of relatively high molecular weight and low volatility, accumulated. It was noted that the number of mushrooms increased with greater depths of casing and that sporophore initiation could occur on heavily watered compost. Both situations were envisaged as leading to greater trapping of the "hormones" but no experimental proof of their existence was provided.

In view of the interest in volatiles from mushroom mycelium, both for their possible effects in initiation and their role in basidiospore germination (McTeague, Hutchinson and Reed, 1959; Lösel, 1964; Lockard, 1962); Lockard and Kneebone (1962) made an extensive study of the gases derived from cultures grown under aseptic conditions. Sporophores were grown in large aerated flasks, the effluent gases being sampled when required for analysis by gas chromatography and infra-red spectroscopy. Ethylene, acetaldehyde, acetone, ethanol and ethyl acetate were identified while small amounts of unidentified higher molecular weights substances were also detected. Tschierpe and Sinden (1965) found that production of these volatile materials was influenced by environmental conditions. In air containing more than 4% oxygen vegetative mycelium produced oxalic acid, CO_2 and possibly acetaldehyde, while sporophores released only CO_2. At a lower oxygen tension, i.e. 2%, the mycelium evolved the typical fermentation products CO_2, ethanol, acetaldehyde and ethyl acetate, while sporophores produced ethanol in addition to CO_2. With the exception of CO_2 none of these volatile

compounds have been assessed for their morphogenetic effects on A. bisporus. Ethanol is known to induce rhizomorph formation in Armillaria mellea (Weinhold, 1963; Pentland, 1967) and acetaldehyde has been included among the sporostatic factors functioning in fungi (Robinson and Park, 1966). Ethylene is biologically active in fruit ripening (Biale, 1960), flower wilting (Smith, Parker and Freeman, 1966) and plant pathogenesis (Wood, 1967).

Mushroom growers have been aware of the adverse physiological effects of high CO_2 tensions on fruit body development since Lambert's (1933) studies, but its central role in sporophore initiation was not established until the work of Tschierpe (1959). In experiments using small, forcibly aerated, sealed chambers it was demonstrated that concentrations above 0.5% CO_2 in air over the casing surface inhibited initiation and led to extensive development of vegetative mycelium. Interpreting these results and other observations in growing houses, Tschierpe postulated that initiation was a response to a CO_2 concentration gradient, existing within the casing layer, produced by high respiratory CO_2 levels in the underlying compost and relatively low atmospheric CO_2 tensions above the casing. Tschierpe claimed that all the effects ascribed to unknown volatiles by earlier workers could be produced in his apparatus by alterations in the CO_2 levels of the ventilating air stream. Evidence reinforcing the CO_2 gradient hypothesis was presented by Tschierpe and Sinden (1964), employing larger growth chambers which permitted recording the number and weight of sporophores produced and analysis of the effluent gases. Optimal CO_2 concentrations for fruit body production were in the range 0.03% (the lowest level

examined) to 0.1%. Exposure of hyphae at the surface of casing to these levels for as little as 24 hours was sufficient to initiate formation of mushroom primordia. No evidence was found for the participation of other volatile controlling factors.

It has been established that casing factors other than CO_2 play a role in fruit body formation of A. bisporus. Trials with various casing soils led Pizer and Leaver (1947) and Borzini (1949) to postulate the existence of relatively immobile chemical factors. Later, after extensive experimentation, Eger (1959, 1961, 1962a, 1965 a,b) suggested that micro-organisms were implicated in the initiation process. This worker demonstrated the supression of initiation in autoclaved casing which was overcome by addition of a soil suspension taken from around already formed primordia. The activity of the soil suspension was annulled by Seitz filtration, indicating that water soluble factors were probably not involved. Initiation also followed exposure of sterilised casing to the growing room atmosphere and could occur when soil suspensions were added to a foam-like plastic material substituted for the normal peat and loam casing mixtures. Attempts to obtain the specific organisms responsible were frustrated through the rapid loss of activity by the isolates (Eger, personal communiation), but initiation was induced by a mixed actinomycete/bacterial culture. However, small numbers of sporophores were consistently formed in sterile activated charcoal used alone or mixed with soil to increase its water holding capacity. Eger (1962b) demonstrated that mushroom mycelium produced volatile antagonists to the growth of a variety of soil micro-organisms and concluded that initiation was

a response to interaction between the mushroom mycelium in the casing and a specifically selected microflora. The microbes might function through the removal of substances from the vicinity of the hyphae, as suggested by the findings with activated charcoal (Eger, 1961, 1965b).

Tschierpe and Sinden (1964) questioned Eger's findings and suggested that microbial action might merely represent detoxification of autoclaved casing. Alternatively (personal communication) these workers have proposed that re-synthesis of heat labile growth factors could account for the apparent involvement of micro-organisms in sporophore initiation.

Additional evidence supporting Eger's findings has been provided by O'Donoghue (1962) who observed sporophore formation in a bottle of mushroom grain-sprawn associated with actinomycete contamination. Also, Thomas, Mullins and Block (1964) and Hayes (personal communication) have consistently failed to produce mushrooms employing sterile substrates: while San Antonio (personal communication) was unable to produce sporophores in large scale gnotobiotic culture. It is interesting to note in this context that Urayama (1960) obtained an isolate, designated Bacillus psilocybe which stimulated increased fruiting in Psilocybe panaeoliformis cultures, and in a number of other agarics, including A. bisporus (Urayama, 1961). With A. bisporus a suspension of the organism was sprayed on to unsterilised compost and casing. An unidentified active substance was extracted from cultures of the bacterium (Urayama, 1960), but the organism became inactive before characterisation could be completed (Edwards, personal communication).

Several workers have claimed that sporophores of the cultivated mushroom can be produced in sterile culture using normal casing materials (Schisler, 1957; Koch, 1958; Lockard, 1962; Lockard and Kneebone, 1962), but their results were not substantiated by other workers Eger (1961) and Thomas et al (1964).

The observations and conclusions of the latter workers using petri-dishes subdivided into four quadrants by low internal partitions, permitting physical separation of compost and casing, are particularly significant. One quadrant of a series of dishes was filled with spawn run compost from which hyphal strands invaded sectors containing sterile casing soil. Sporophores developed only when the sterile casing was inoculated with soil suspensions taken from around earlier formed fruit bodies, supporting Eger's (1961) observations. Because of the separation of compost and casing it was claimed that no CO_2 diffusion gradient (c.f. Tschierpe, 1959) could exist. Also since any mycelial volatiles produce were equally present over both inoculated and uninoculated sectors, without inducing carpogenesis in the latter, it was unlikely that these were involved in the initiation process.

Lipids, possibly sterols, may have an essential role in mushroom sporophore initiation (Schisler and Sinden, 1966; Schisler, 1967). When vegetable oils such as cotton seed oil or groundnut oil were added to spawn run compost at casing, increased yeilds resulted due to production of larger numbers of sporophores in the first two flushes of the crop. As there was no concurrent increase in compost temperature, this was taken as evidence for incorporation of the lipid, rather than its utilisation as an energy source.

The aim of the present study was to provide an explanation for the specific function of the casing layer in muchroom culture. Attention was concentrated principally on carbon dioxide control and microbial involvement as these views appeared most firmly based on experimental evidence. The role of lipids and other factors was also examined.

MATERIALS AND METHODS

Mushroom strains

Commercial white (White Queen 101, Mounts' White, Sinden White and Somycel 59) or cream (Sinden Cream) strains of Agaricus bisporus were examined as indicated in the text.

Growth media

Three media were employed for mycelial growth of Agaricus bisporus during this investigation.

a) Malt extract agar : 2% malt extract (Oxoid), 1.5% agar (Oxoid No. 1), pH 6.0, sterilised by autoclaving at 121°C. for fifteen minutes. This medium was used for maintenance of laboratory isolates, which were grown on slopes at 25°C. for fourteen days and stored at room temperature. Subculturing was performed at six monthly intervals.

b) Unsterilised horse-manure compost, prepared at Wrington Vale Nurseries Ltd., using the "short composting method" (Sinden and Hauser, 1950). For convenience spawn-run compost was utilised, prepared commercially by inoculating prepared compost with 2-3 oz. grain spawn per cubic foot and incubating at 22-27°C. (72°-80°F.) for twelve to fourteen days.

c) Sterilised compost, prepared by autoclaving 200 g. quantities of commercial unspawned compost at 121°C for one hour on three successive days. Each batch of compost was inoculated with 20-30 5 mm. diameter discs cut from a 7-14 day old plate culture of Agaricus bisporus and incubated at 25°C. for three to four weeks. The fully colonised compost was stored at 16.5°C for up to two months prior to use.

Casing materials

The casing materials described below were used for sporophore

production in conjunction with the compost media.

a) A 4:1 (volume/volume) mixture of Irish sphagnum peat or Somerset sedge peat and ¼" grade Carboniferous limestone chippings (final pH 6.8-7.0) adjusted to 90% of its maximum water holding capacity with distilled water.

b) An alkaline clay-loam soil (pH 7.0-7.2) obtained from the University grounds and similarly adjusted to 90% of its maximum holding capacity.

c) The following proprietary materials:

¼" grade "Diatomite" an expanded silicate (Moler Products Ltd.) pH 6.0; acid washed sand (British Drug Houses) pH 6.0 and gas adsorbent charcoal (British Drug Houses) pH 7.5. These materials were saturated with distilled water prior to use.

Where required casing materials were usually sterilised by autoclaving in 200 g. aliquots at 121°C. for one hour on three successive days. Alternatively 25 g. quantities were exposed to 15 ml. propylene oxide in a vacuum oven (capacity 2 litres) at a pressure of 100 mm. Hg. and temperature of 37°C. for sixteen to twenty-four hours. At the end of this period remaining traces of propylene oxide were removed by repeated flushing with a stream of bacteriologically filtered carbon dioxide.

Production of sporophore initials

Eger's (1959, 1961) half-plate technique ("Halbschalentest"), fig. 1, was adopted for preliminary screening of experimental materials. One half of a petri-dish base was filled with approximately 10 g. spawn-run compost and the adjacent half with 20 g. peat casing or an equivalent volume of the other materials. Sufficient space was allowed between the surface of the contents and the dish lid to permit gaseous

Fig.1. The Half-plate technique (Halbschalentest)
The lower half of the petri-dish contains unsterilised spawn-run compost;the upper part unsterilised peat casing.

exchange. Similarly filled transversely or "Y" partitioned plates were also employed. Test suspensions or solids were added to the centre of the casing adjacent to the compost, distributed uniformly throughout the casing or compost, or placed upon the hyphae advancing into the casing. Results were expressed as rate of mycelial growth into the casing and as numbers or frequency of sporophore initials. All treatments were replicated ten times except where large numbers of tests were simultaneously performed when two or three replicates were employed.

In practice it was found that considerable variation occurred in some of the controls. Other methods were tried but none proved superior to the half-plate technique. Because of the inherent defects in the method the findings could not be interpreted rigidly but they nevertheless provided useful information concerning the effects of additives on vegetative growth and sporophore initiation.

For more controlled experimentation vertically mounted tubular growth chambers, Fig. 2, were utilised. These consisted of 3.6 x 20.0 cm. pyrex glass tubes, fitted at each end with rubber bungs (B.S.33) perforated by 7 cm. lengths of 7 mm. diameter glass tubes for air entry and exit. Approximately 30 g. spawn-run compost was compressed into the basal 8 cm. of the chamber and covered with a 2 cm. layer of casing material. The chambers were aerated by passing laboratory air or gas mixtures of defined composition (British Oxygen Ltd.) downwards through the casing and compost respectively, Fig. 3. Laboratory air was drawn through the apparatus under slight negative pressure by oil-free diaphragm pumps (Type Mu 19/30 or 19/65, Charles Austen Ltd.) which could each accommodate up to eight growth chambers. Air flow through individual chambers was controlled by Hoffman clips

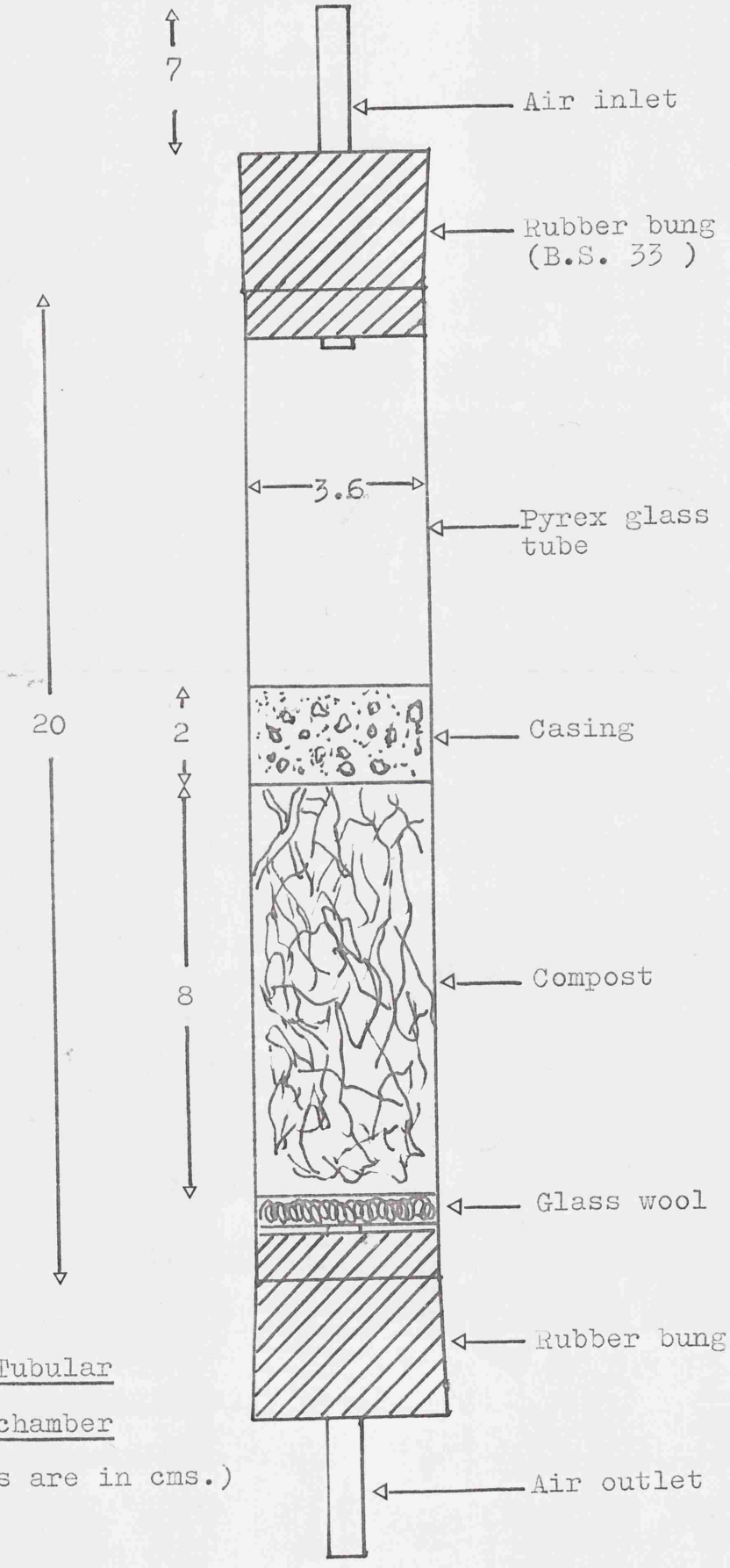

Fig.2. Tubular Growth chamber

(Figures are in cms.)

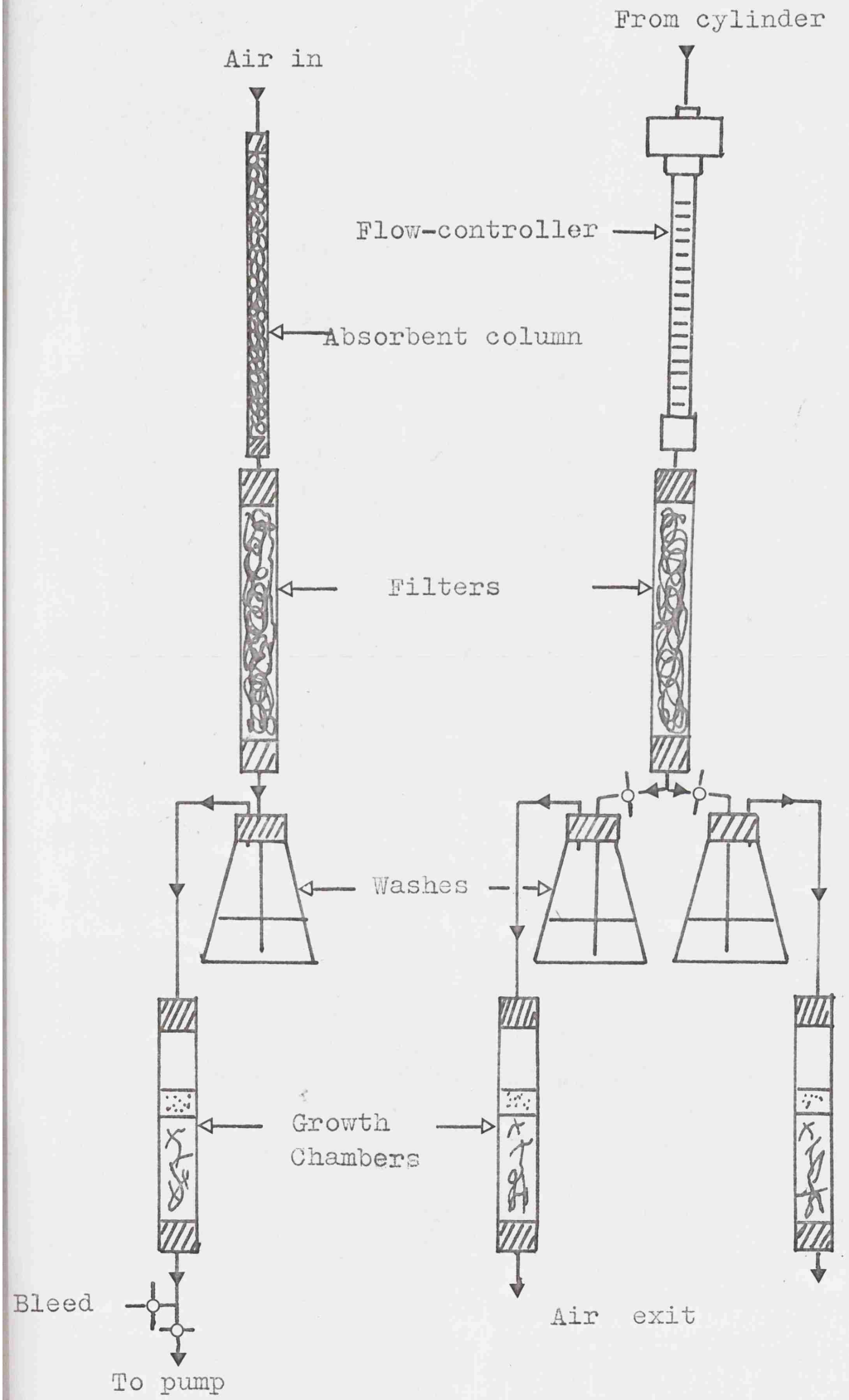

Fig. 3. Air flow to culture chambers.

and a bleed valve positioned between the chamber and the pump. Gas mixtures were metered under slight positive pressure from 220 cu. ft. capacity cylinders via flow-controllers (G.A. Platon Ltd.) to two or four growth chambers. The flow rate for both types of aeration was 50 ml. per minute per chamber. Before entering the chamber the air or gas mixtures were humidified to at least 80% R.H. by passage through a distilled water wash. All connections between growth chambers, humidifying flasks, pumps and ancillary equipment were of 6 mm. internal diameter latex tubing (Gallenkamp). To ensure adequate seals the rubber to glass joints were bound with copper wire, while the tops of the wash bottles and growth chambers were coated with paraffin wax.

For pure culture studies a 3.6 x 30.0 cm. non-absorbent cotton-wool filter was inserted in the flow-line prior to the humidifying flask. The filter and all subsequent components were sterilised separately by autoclaving before filling the chambers and assembly. When required 2.0 x 27.0 cm. columns of the appropriate gas absorbent were positioned on the inlet side of the filter. The apparatus required little attention while functioning, apart from daily checks and occasional adjustment of the flow rates while its use permitted close control of the casing atmosphere. In addition to observations on sporophore development, daily measurements of hyphal growth into the casing layer were made at four points 90^{o} apart using vernier callipers. All treatments were duplicated, including positive or negative controls as necessary and findings were confirmed by repetition. The use of these growth chambers overcame many of the difficulties associated with the half-plate technique and permitted a high degree of reproducibility.

All experiments involving production of sporophores were performed in a growth room operating at between 15.5° and 18.0° C.; a range approximating to that prevailing under commercial growing conditions. Individual experiments lasted 14 to 21 days and were occasionally extended to 35 days.

EXPERIMENTAL

Several approaches were followed in an attempt to elucidate the factors affecting sporophore initiation and related phenomena in Agaricus bisporus. For convenience these are considered separately under the sub-headings below. The White Queen strain of A. bisporus was used, unless otherwise stated.

1. The effect of Carbon Dioxide.

a) In unsterilised substrates

The work of Lambert (1933); Tschierpe (1959) and Tschierpe and Sinden (1964) stresses the central role of carbon dioxide in sporophore formation and development in A. bisporus. The CO_2 gradient hypothesis proposed by Tschierpe (1959) was based on observations of the effects of various CO_2 concentrations overlying the casing. High CO_2 concentrations have been reported in spawn run compost (Tschierpe, 1959; Rasmussen, 1962) and there can be no doubt that a CO_2 concentration gradient exists in the casing layer. However, these gradients have not been measured and the behaviour of mycelium within the casing layer under defined CO_2 concentrations has not been examined, presumably because of technical difficulties. Measurement of CO_2 in casing would be particularly difficult under commercial growing conditions since the CO_2 gradient would fluctuate as a function of the respiratory rate of the mycelium in the underlying compost, which is known to vary considerably during the developmental cycle (Schisler, 1957; Tschierpe, 1959 and Tschierpe and Sinden, 1964). In the experiments described below metabolic CO_2 was flushed away from the casing in growth chambers, permitting the establishment of defined, uniform concentrations of the gas within this layer. With all experiments in this phase of the study the growth chambers were filled with

spawn run compost overlain by Irish sphagnum peat casing.

Initially the effect of removing CO_2 from the casing layer was examined. Laboratory air was passed through self-indicating soda-lime columns to remove CO_2 and then drawn down through the chambers, except in one set where the flow was in the reverse direction. The control chambers were supplied with downward moving untreated air. After 14 days mycelial strands had grown into the casing in the controls and formed initials. Downward moving CO_2 free air completely inhibited hyphal growth and sporophore initiation in the casing (Fig. 4). In the upward flowing airstream hyphal growth into the casing was dense and only a few poorly formed primordia developed. The experiment was repeated with identical results, while in subsequent studies the treatments employing downward flowing air were replicated at least twenty times without divergence from the original observations.

Before these results could be interpreted it was considered necessary to test the experimental system further, in view of the report by Nichols and Topping (1966) that self-indicating soda-lime could remove ethylene, probably through the activity of the manganate ion of the indicator. Three gas absorbents were examined; self-indicating soda-lime (B.D.H.), white soda-lime (B.D.H.) and gas adsorbent charcoal (B.D.H.). Hyphal growth and sporophore formation were suppressed when employing both forms of soda-lime, but were unaffected by air which had passed through gas adsorbent charcoal. These results indicated that CO_2 was the active atmospheric component removed by the soda-lime.

In another experiment four chambers were supplied with soda-lime

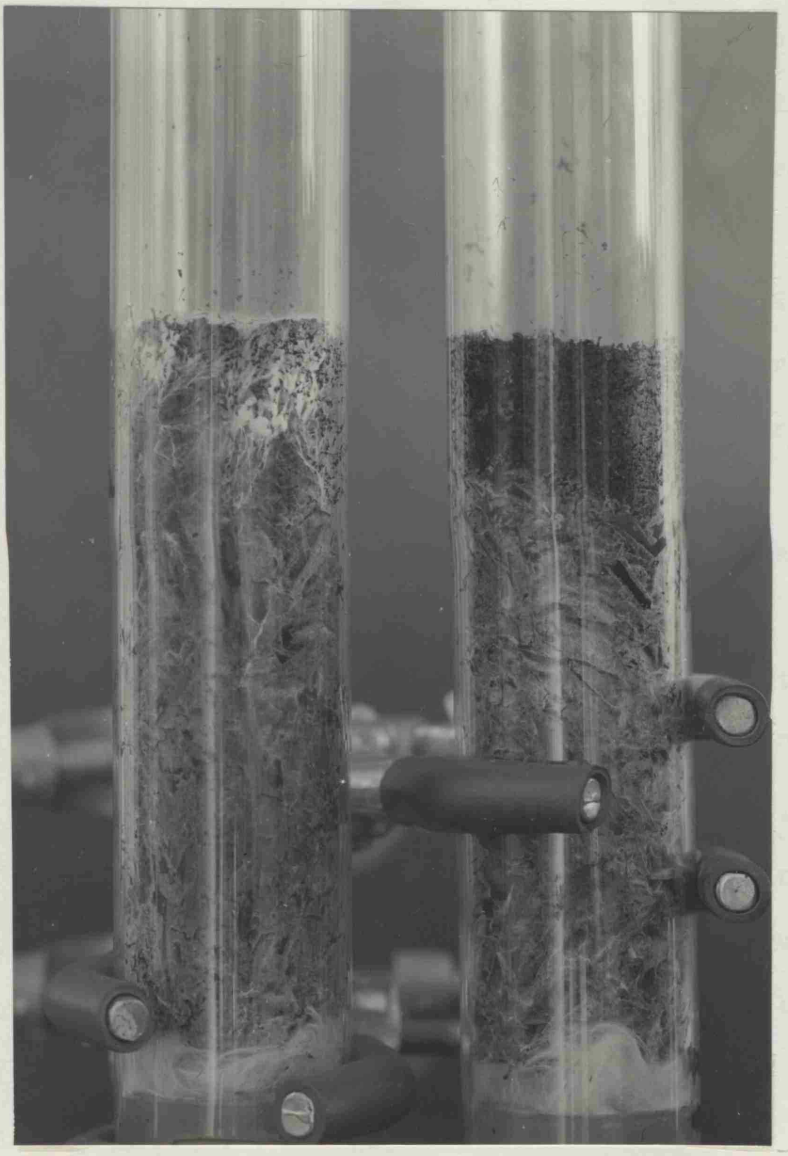

Fig.4. The effect of CO_2 free air in unsterilised sphagnum peat casing, after 14 days. Left chamber , untreated control with well developed hyphal strands and initials . Right chamber , CO_2 free air , no strand growth in the casing and no initials.

treated air for 13 days. The soda-lime columns were then removed and aeration with laboratory air continued for a further 22 days, resulting in growth of mycelial strands into the casing and formation of mushroom primordia which subsequently developed into mature sporophores (Fig. 5). Thus lack of CO_2 had no permanent effect on hyphal development. The time required for sporophore initiation paralleled that prevailing under commercial conditions for the mushroom strain employed, indicating that the apparatus functioned satisfactorily in this respect. Carbon dioxide is thus required for growth of mycelium of A. bisporus, but a CO_2 gradient is not a prerequisite for carpogenesis, contrary to Tschierpe's (1959) interpretation. Sporphore formation, however occurred principally within the casing layer and not at the surface as in commercial production, suggesting that CO_2 concentration may influence the position at which initiation occurs.

The effects of a range of CO_2 concentrations was next examined using defined air mixtures of $\lesssim$ 1, 22, 42, 104, 240, 340, 1000, 3,000 and 6,700 p.p.m. CO_2, metered from cylinders. Unfortunately for technical reasons only three concentrations could be examined simultaneously. While care was taken to use 14 day old spawn-run compost and the same type of casing throughout, slight variations between batches of compost were unavoidable. The results obtained are expressed graphically in Figs. 6 and 7.

Although maximal growth rates were attained with 3,300 and 6,700 p.p.m. CO_2, rates over the first five days were closely parallel over the range 104-6,700 p.p.m. CO_2. Thereafter, between 104 and 1000 p.p.m. a growth retardation occurred associated with the onset of initiation. At concentrations higher than 1000 p.p.m. the early growth

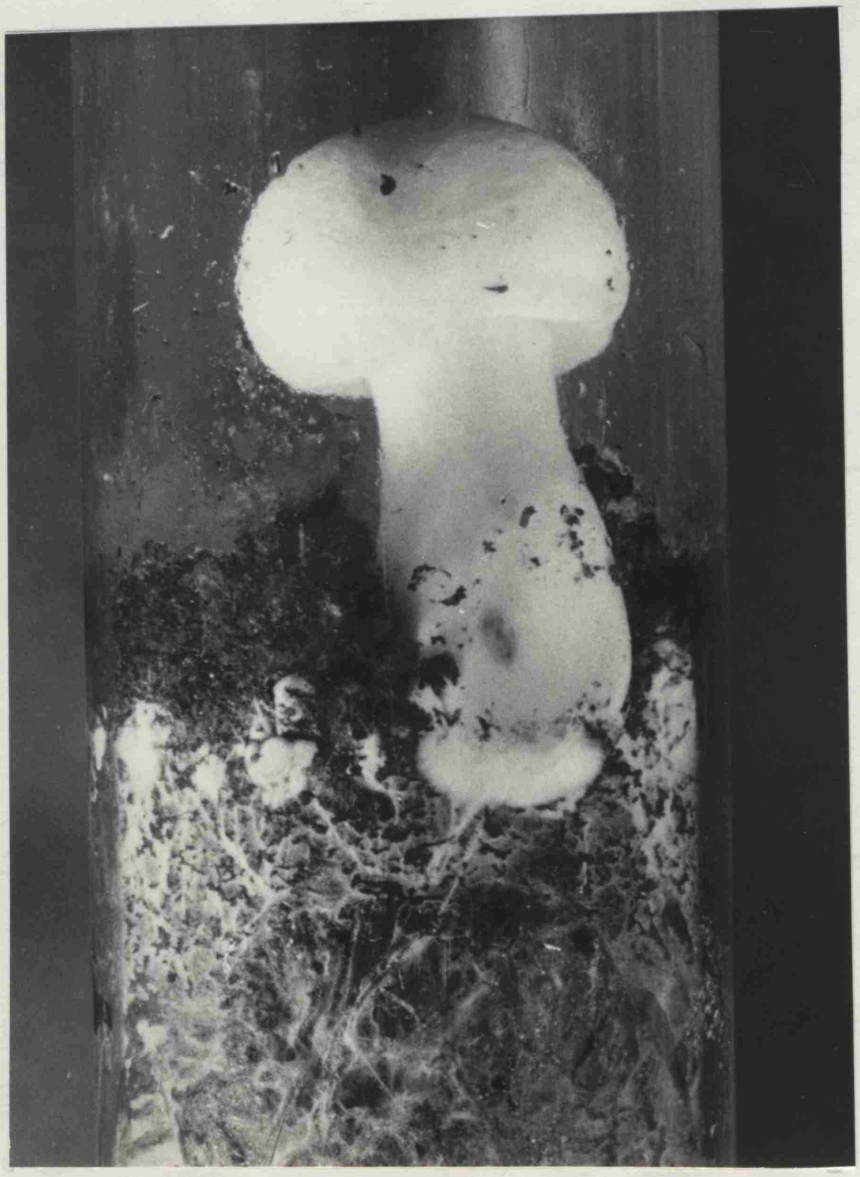

Fig.5 . Sporophore development after 21 days in untreated air .

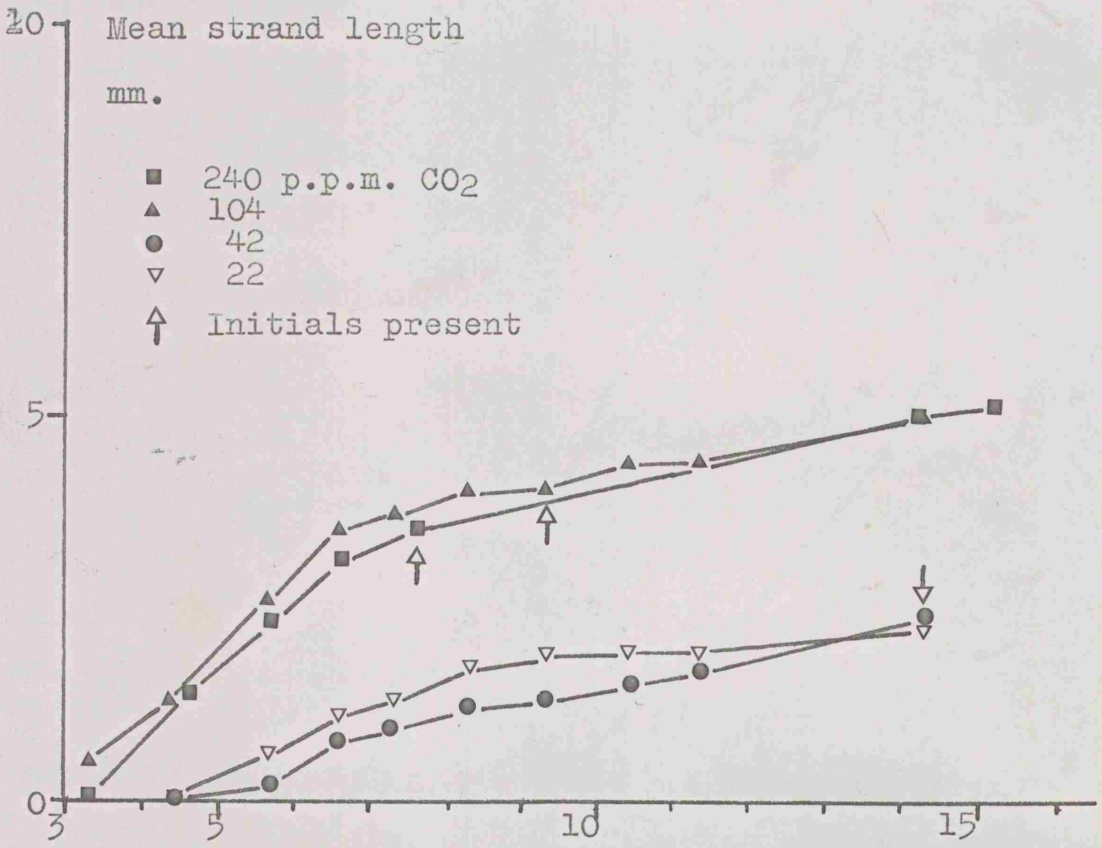

Fig.6. Strand growth into unsterilised casing at 22;42;104 and 240 p.p.m. CO_2.

No growth into the casing took place with < 1 p.p.m CO_2 .

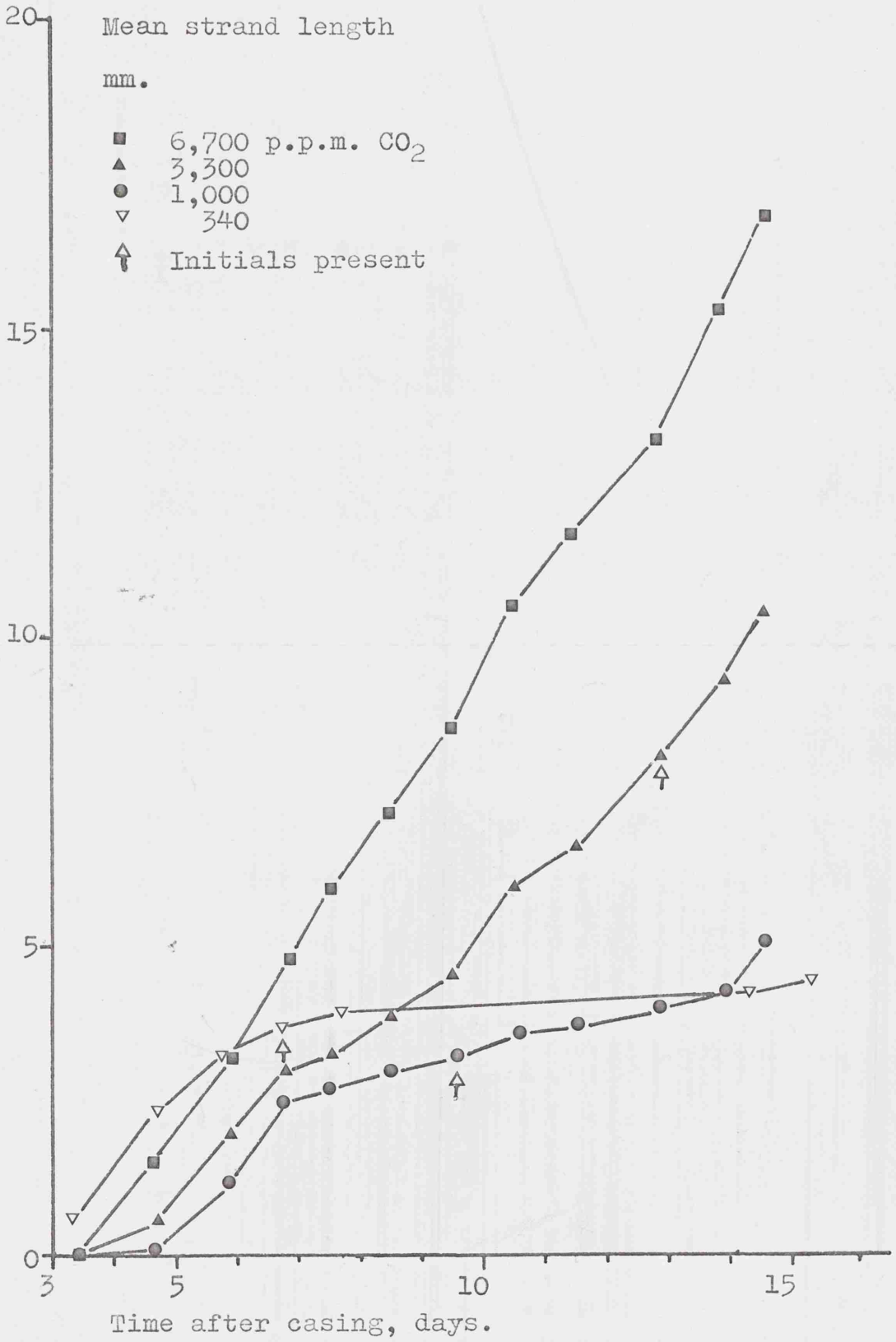

Fig. 7. Strand growth into unsterilised casing at 340;1,000;3,300 and 6,700 p.p.m. CO_2 _

rates were maintained throughout the experiment associated with a marked decline in primordia formation. Initial growth rates at 22 and 42 p.p.m. were reduced suggesting that these concentrations were suboptimal for vegetative growth. The reduction of growth rates at these concentrations was also less pronounced, occurring after the eighth day. No growth or initiation occurred at less than 1 p.p.m. CO_2. Hyphal growth into the casing at 11 days is plotted as a function of CO_2 concentration in Fig. 8, which further emphasises the retardation in growth between 104 and 1000 p.p.m.

The inhibition of mycelial growth into casing in the above experiments when using 1 p.p.m. CO_2 confirms that efficient removal of metabolic CO_2 at the airflow rate employed. Volatile materials other than CO_2 produced either in the compost or casing would also be displaced and are thus unlikely to be involved in sporophore initiation.

Carbon dioxide also exerted a controlling effect upon sporophore initiation. Most sporophores (95%) were formed in the range 104-1000 p.p.m. (Table 1), 68% of these being produced at concentrations between 340 and 1000 p.p.m. confirming previous observations by Tschierpe and Sinden (1964). Within the latter range primordia formed earlier and developed faster than at other concentrations, suggesting that CO_2 might be essential for carpogenesis. This possibility was confirmed by three experiments employing laboratory air, of twenty-one days duration. In the first of these Mount's White Strain was subjected to untreated air for 7 or 14 days after which the incoming air was passed through soda-lime columns. Development of initials, formed on days 5 or 6 was arrested when the CO_2

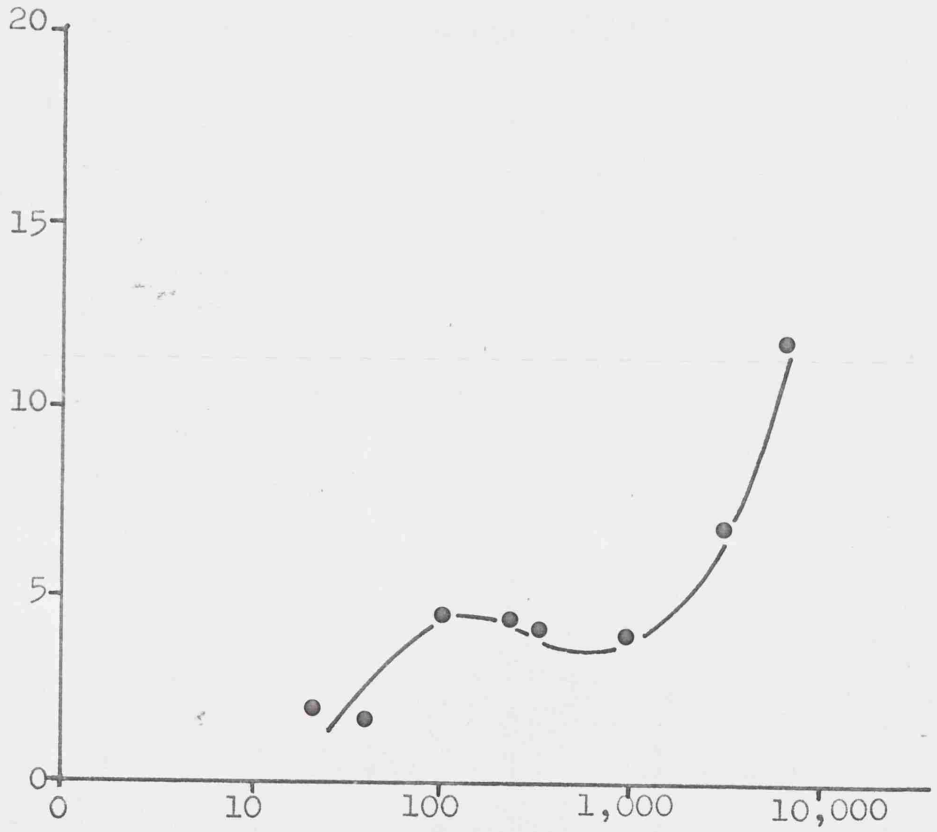

Fig. 8. The effect of CO_2 on strand growth into unsterilised casing.

CO_2 level in p.p.m.	Numbers of initials formed up to 14 days from casing.			Time to first initials (Days)	Time to first pinheads (5m.m.) (Days)
	Chamber 1	Chamber 2	Total %initials formed		
1	0	0	0	-	-
22	1	0	0.5	14	-
42	2	3	2.9	14	-
104	7	10	9.7	-	-
156	6	2	4.6	10	-
240	15	7	12.6	7	15[1]
340	16	15	17.7	7	11
370	14	12	14.9	9	12
1000	11	14	14.3	7	10[2]
1000	16	21	21.2	9	12
3,300	1	2	1.7	12	-
6,700	0	0	0	-	-

Note 1. This was a 21 day experiment.

2. Full sporophore development did not take place.

Table 1 Effect of CO_2 concentration on sporophore initiation and development in non-sterile materials.

supply was interrupted after 7 days but continued normally after cessation of CO_2 at 14 days. A similar experiment with the Sinden White strain confirmed the CO_2 requirement for development of initials; no growth of primordia taking place following introduction of CO_2 free air in the fifth or tenth days after casing. From a third experiment, employing the White Queen 101 strain it appears that CO_2 was necessary for the initiation process. The CO_2 supply was halted after 4, 6, 8, 10, 12 and 14 days with the results given in Table 2. Only in chambers receiving 14 days or more normal air did development of initials occur. No initials formed in this experiment unless CO_2 was supplied for 8 days. It would seem that once a sporophore commences its development it quickly becomes independent of an external CO_2 source, probably through increase endogenous production of this metabolite.

While the data presented above clearly indicated an overall control by CO_2 on hyphal growth and sporophore initiation, it was not the sole external factor inducing the latter process. When an equivalent amount of unspawned compost was substituted for peat as a casing material a different response occurred. The results of such an experiment employing air mixtures of <1, 12, 124, 1000 and 11000 p.p.m. CO_2 have been recorded in Figs. 9 and 10. Growth of hyphae was related to CO_2 concentration with a progressive delay in the onset of linear growth at the lower levels employed. No checking of hyphal growth occurred at any of the CO_2 levels and no initials developed. Nutritional factors can thus modify the response of hyphae to CO_2 and suppress sporphore formation.

b) <u>In sterilised substrates</u>

Further evidence for the operation of other factors in the

Duration of Normal CO_2 supply (Days)	Developmental stage at cessation of CO_2 supply		Developmental stage at end of experiment (23 days)	
	Chamber 1	Chamber 2	Chamber 1	Chamber 2
0	No strand growth			
4	0	0	0	0
6	0	0	0	0
8	I*1	I	V*2	I
10	I	I	I	I
12	I	I	I	I
14	I	II	V	V
23	-	-	I	V

*1 These initials were well formed.

*2 Very stunted and malformed.

Developmental Stages

0 = No initials produced

I = Initial

II = Initial/Pinhead (2 mm)

III = Pinhead (5 mm)

IV = Pinhead/Button

V = Button (10 mm)

VI = Cup

Table 2. Effect of cessation of CO_2 supply

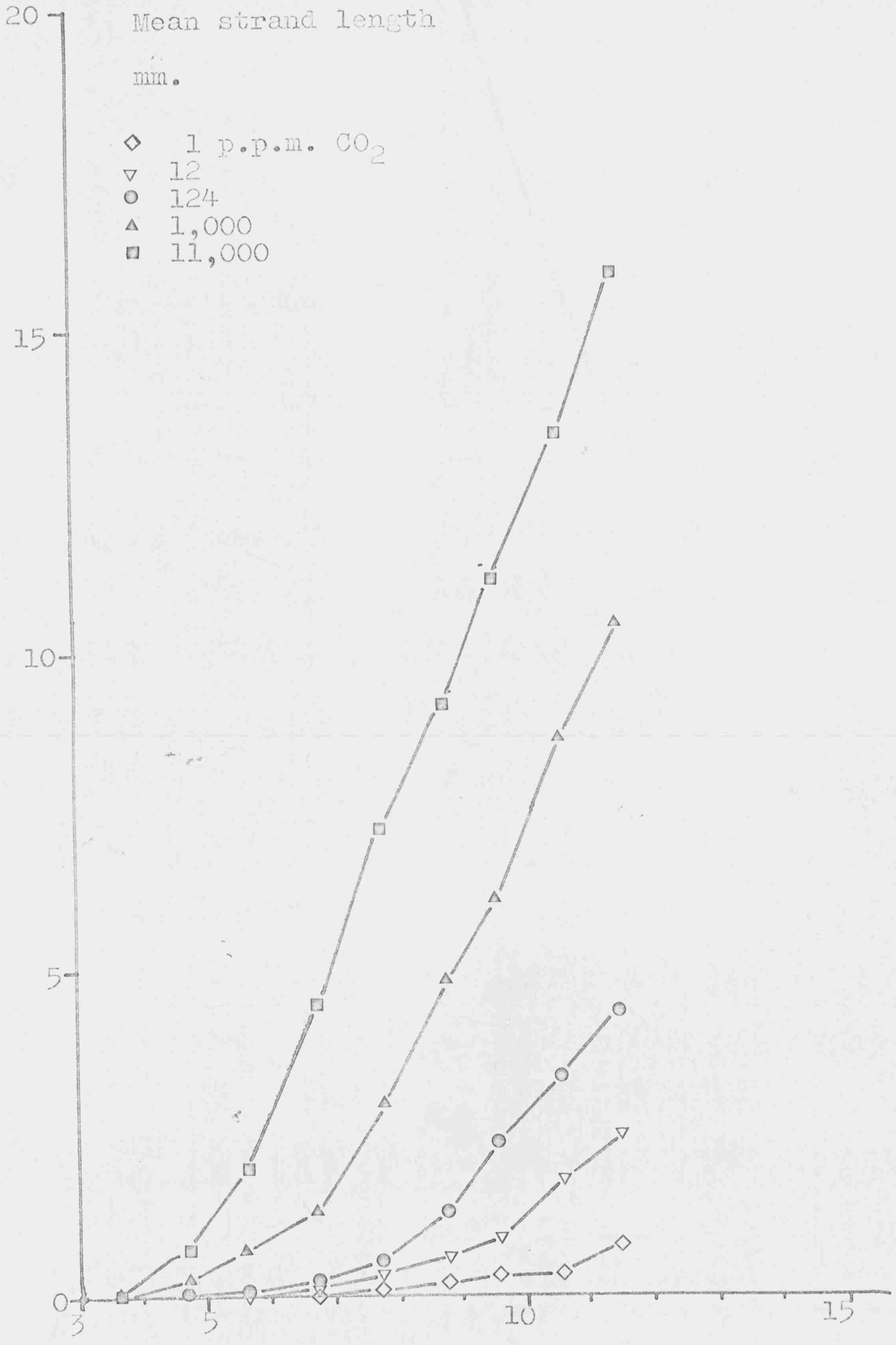

Fig.9 . Growth of hyphal strands into unsterilised compost at 1; 12;124;1,000 and 11,000 p.p.m. CO_2.

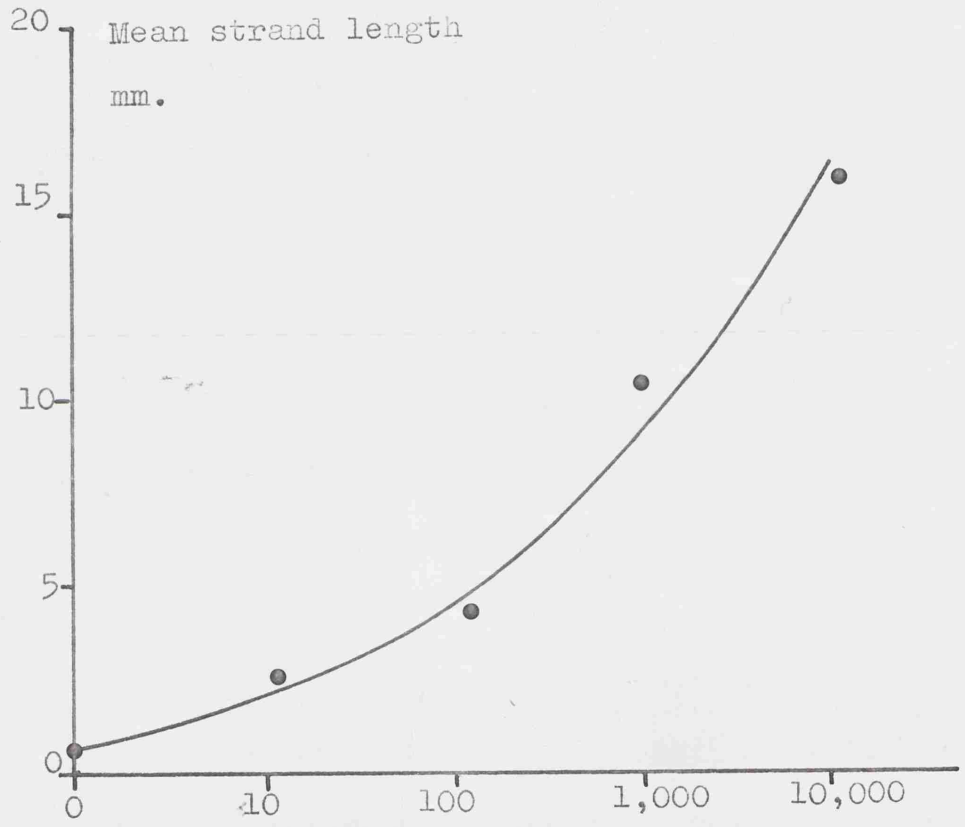

Fig. 10. The effect of CO_2 on strand growth into unsterilised compost.

initiation process was provided by results from experiments employing autoclaved compost inoculated with the White Queen 101 strain and autoclaved Irish sphagnum peat casing in the growth chambers. The data obtained when these materials were subjected to 22, 42, 104, 156, 370, 1000, 3,300 and 6,700 p.p.m. CO_2 has been presented in Figs. 11, 12 and 13. Hyphal strand growth into the casing was rapid and dense compared with that observed in non-sterile materials, and increased in proportion with CO_2 concentration up to 370 p.p.m. At this concentration the rate was equivalent to that attained when employing 6,700 p.p.m. CO_2 and non-sterile casing and also compared favourably to the growth rate occurring at 11,000 p.p.m. using compost as a casing material. Above 370 p.p.m. no further increase in growth was observed, presumably due to factors other than CO_2 becoming rate limiting. Sporophore formation did not occur at any of the CO_2 levels employed. Confirmation of these results was obtained using compost and casing from the same source treated with CO_2 levels of $\lesssim 1$, 48, 200, 340, 470 and 700 p.p.m. Duplicate chambers were filled with sterilised, pure culture spawn-run compost covered with autoclaved or untreated peat casing. At all levels above $\lesssim 1$ p.p.m. the differential growth rates observed previously in sterile and non-sterile casing were maintained, an example being given in Fig. 14. Initial formation, however, was delayed, while clearly recognizable primordia occurred only in unsterilised casing at 700 p.p.m. CO_2.

Tschierpe and Sinden (1964) passed air streams of appropriate CO_2 content over hyphae near the surface of casing to demonstrate sporophore induction after 24 hours. Two attempts were made to repeat this procedure with pure cultures, employing a 1 cm. casing layer to reduce the time hyphae required to reach the casing surface. On both occasions the chambers were subjected to 3000 p.p.m. CO_2 until the mycelium reached the casing surface.

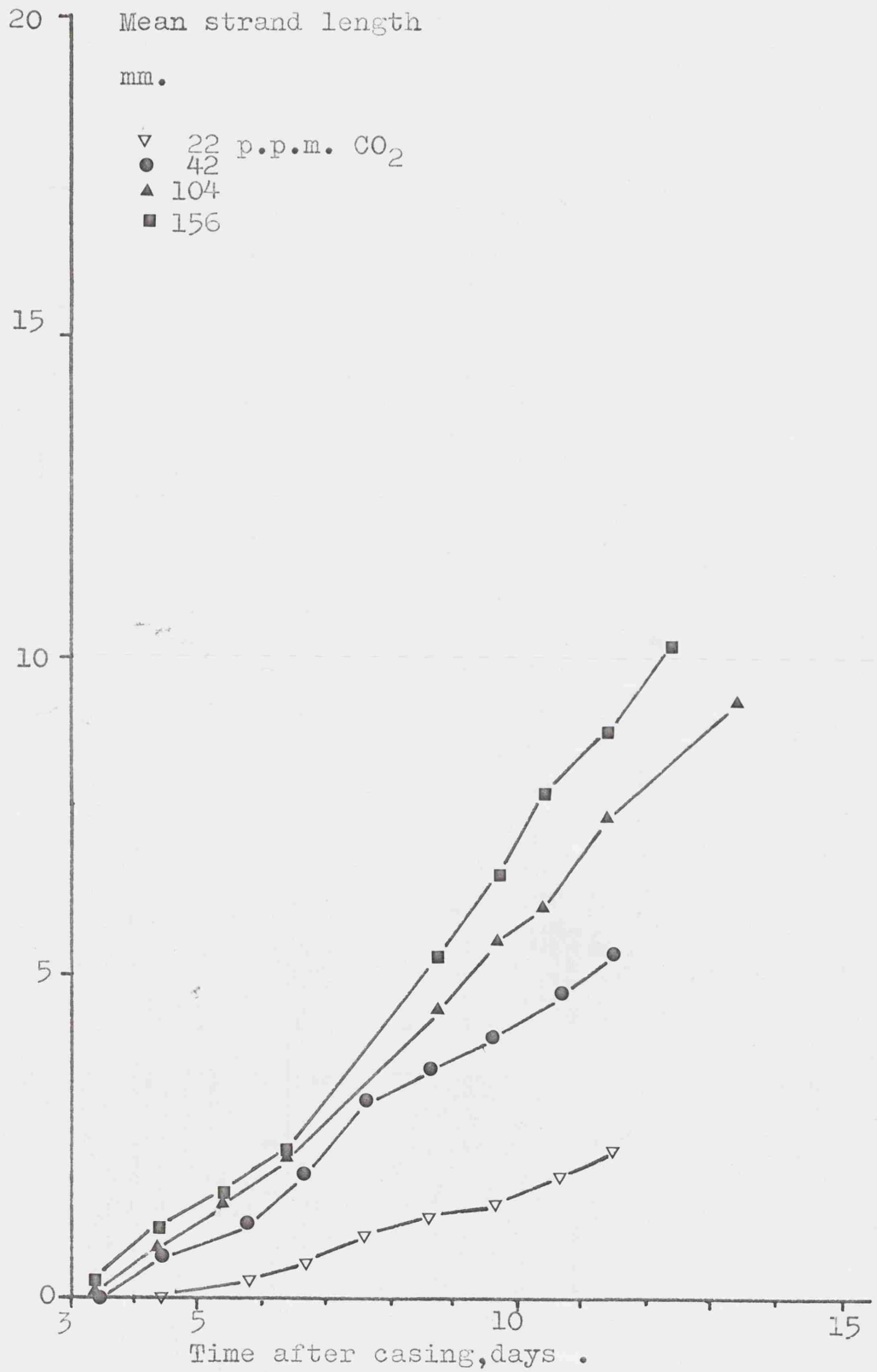

Fig.11. Strand growth into sterile casing at 22;42;104 and 156 p.p.m. CO_2.

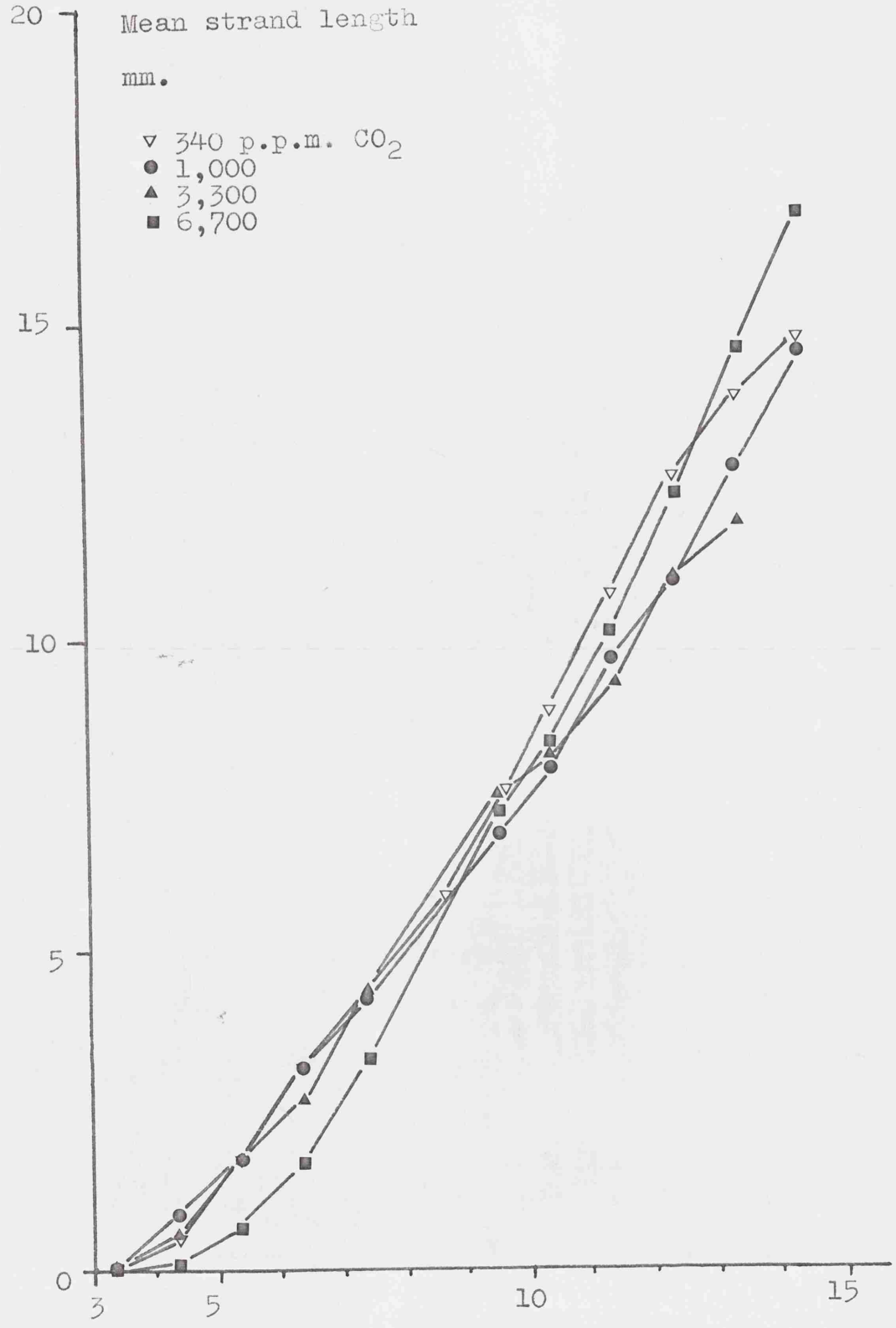

Fig. 12. Strand growth into sterile casing at 370;1,000; 3,300 and 6,700 p.p.m. CO_2

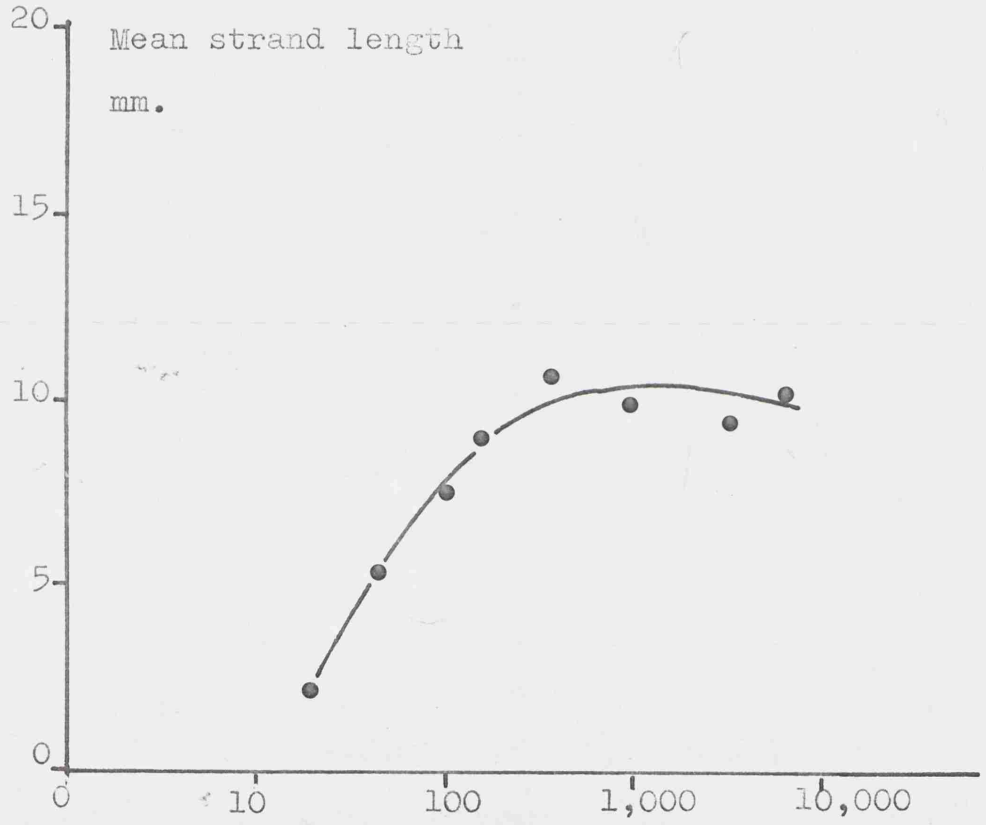

Fig. 13. The effect of CO_2 on strand growth into sterile casing.

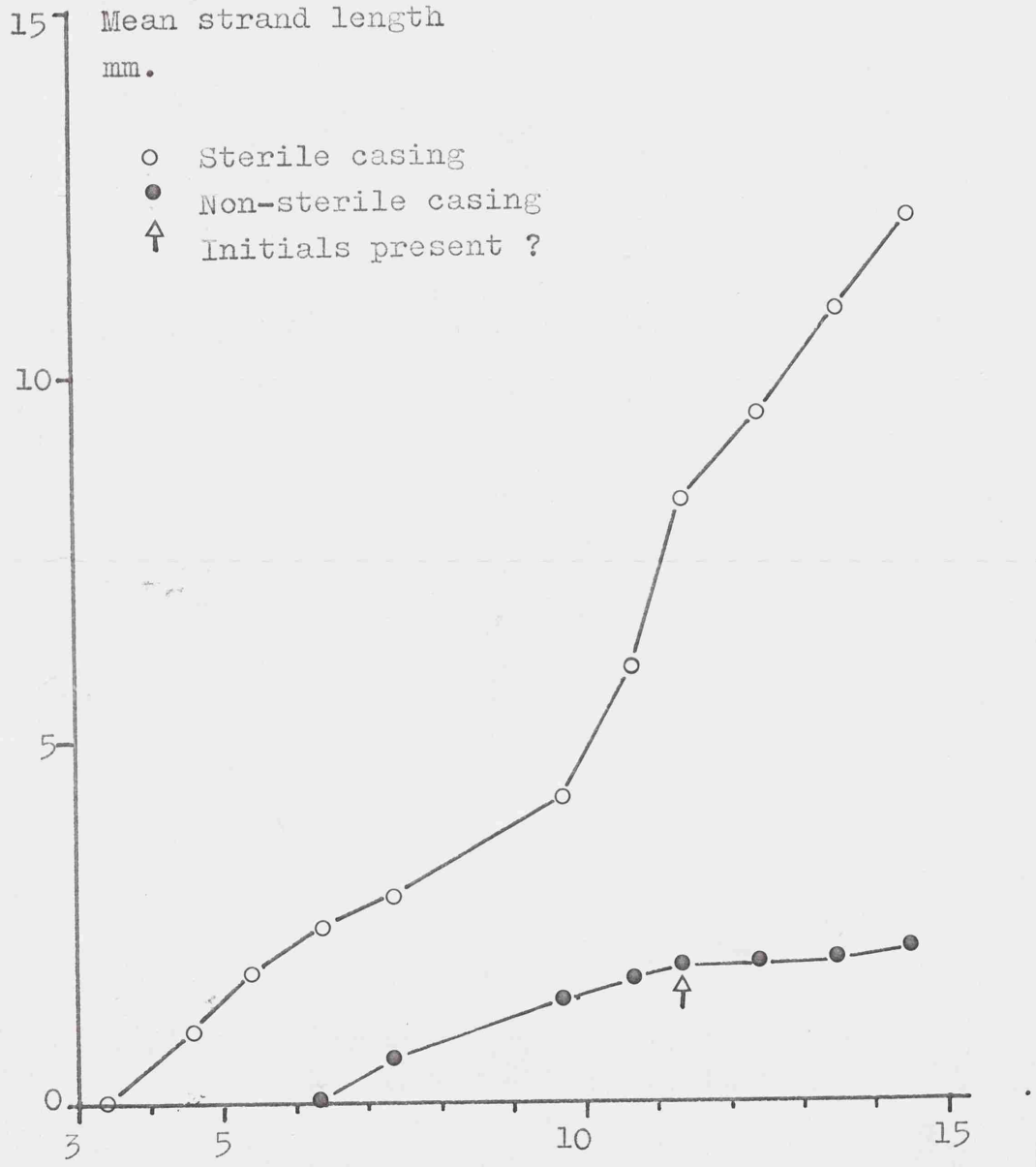

Fig. 14. Strand growth from sterilised compost into sterile and non-sterile casing at 340 p.p.m. CO_2.

The CO_2 levels were then immediately reduced to 170 or 360 p.p.m. and were maintained at these concentrations for 11 days. These treatments failed to promote sporophore formation, hyphal growth continuing above the casing surface in both cases. Initiation did occur when the experiment was repeated with non-sterile materials, changing the CO_2 concentration from 3600 to 360 p.p.m. after 9 days. The results of this control experiment are particularly significant when considered in relation to the CO_2 gradient hypothesis propounded by Tschierpe (1959), In addition to re-emphasising that a CO_2 gradient is not essential for initiation, they demonstrated that CO_2 concentrations above those favouring carpogenesis, i.e. above 1000 p.p.m., are necessary to promote hyphal growth to the casing surface. During commercial mushroom production these requirements are satisfied by levels within the CO_2 gradient in the casing. Thus the CO_2 concentration gradient extant under these conditions permits vegetative growth throughout the casing and this will proceed most rapidly with high CO_2 levels. At the casing surface the over-riding factor inducing maximal initiation would be a change in CO_2 concentration to a level within the range 300 to 1000 p.p.m. Tschierpe (1959) failed to make this distinction and concluded erroneously that initiation was a response to a CO_2 gradient rather than to particular levels of the gas. The gradient hypothesis is still maintained by Tschierpe (personal communication) despite having demonstrated (Tschierpe and Sinden, 1964) the critical CO_2 levels involved.

In view of the retardation of hyphal growth associated with initiation, described earlier, an attempt was made to check mycelial growth in sterile casing by a temporary cessation of CO_2 supply. Cultures were grown for 9 days with untreated air, or without venti-

lation to permit CO_2 accumulation. Both treatments were then ventilated with a CO_2 free air-stream (soda-lime absorbtion) for 72 hours, followed by restoration of the normal air supply. Hyphal growth into the casing was temporarily retarded compared with untreated control but no initials were observed up to 21 days after casing.

The failure to induce hyphal strands to produce sporophore primordia in sterilised materials was open to several interpretations.

a) That micro-organisms or their metabolites in peat casing participated in the initiation process (c.f. Eger, 1959, 1961).

b) Toxic products inhibiting carpogenesis were produce in casing materials as a result of autoclaving (c.f. Tschierpe and Sinden, 1964).

c) The nutritional status of casing was enhanced following autoclaving thereby modifying hyphal responses to CO_2. An analogous situation relating to soil mycortasls has been reported by Ko and Lockwood (1967).

These possibilites are examined in succeeding sections.

Summary

A CO_2 requirement has been demonstrated for hyphal growth, sporophore initiation and the early stages of primordial development in Agaricus bisporus. Mycelial growth rates were faster in sterile than non-sterile casing, increasing with CO_2 concentration up to 370 p.p.m., where it was comparable to that attained at 6,700 p.p.m. in non-sterile casing and at 1000 p.p.m. in compost. The experimental CO_2 levels were arbitrarily selected and no attempt was made to determine the exact optimal CO_2 levels for vegetative growth. Sporophore initiation occurred only in unsterilised materials and was associated with a retardation in hyphal growth. Maximal initiation was observed

over and between 340-1000 p.p.m. in the range of CO_2 concentrations employed. Reduction of hyphal growth in sterile culture did not induce carpogenesis, which was also suppressed by the provision of compost as a nutritional source. Evidence was presented that initiation was partly a response to critical carbon dioxide levels around the hyphal apices, rather than to a carbon dioxide concentration gradient within the casing layer. The failure to produce fruit-body primordia in sterile culture was open to several interpretations; these probably did not include participation by volatiles other than CO_2.

2. The effects of Autoclaving

The participation of micro-organisms in the initiating process (c.f. Eger, 1959-1965b· Thomas et al, 1964) has been confirmed in the present investigation both in half-plate and growth chamber cultures. (Figs. 15, 16, 17 and 18). In spite of many hundreds of attempts sporophores could not be produced in sterilised substrates. Further support for Eger's hypothesis derives from Haye's (personal communication) isolation of a specific bacterium that induces fruiting on a synthetic medium. This may parallel Urayama's (1961) report of the stimulatory effect of Bacillus psilocybe in A. bisporus.

An important observation made by Eger (1961) and confirmed in the present study was the production of primordia in sterile casing overlying non-sterile compost. The inducing factors can thus originate within the compost making it difficult to envisage the agents being other than living microbes. In view of the wide variety of casing materials, soils, peat, vermiculate, coke etc., that have been successfully employed commercially or experimentally it is possible that initiation is not due to specific micro-organisms but is a function of the soil microflora as a whole.

Two experiments with 'Y' or transversely divided petri dishes (Fig. 19) have demonstrated transport of the inducing agents via the hyphal strands, through use of combinations of sterile or non-sterile compost and casing. The results from the 'Y' plate experiment are given in Table 3. In this experiment initiation was confined to 34% of the sterile casing sectors colonised from non-sterile compost. Mixed populations of bacteria were isolated from these sectors. A more restricted flora, qualitatively, was obtained from sectors without initials. The intimate association between bacteria and hyphae which

Fig.15. Sterile half-plate 14 days after filling.

The casing is overgrown with dense, fine hyphal strands.

Fig.16. Sterile half-plate inoculated centrally with 0.1 g. unsterilised casing; after 14 days.

Hyphal strands are thick and relatively few ,initials have formed.

Fig. 17. Aseptic culture chambers after 21 days.
The casing is permeated with a network of dense, fine hyphal strands.

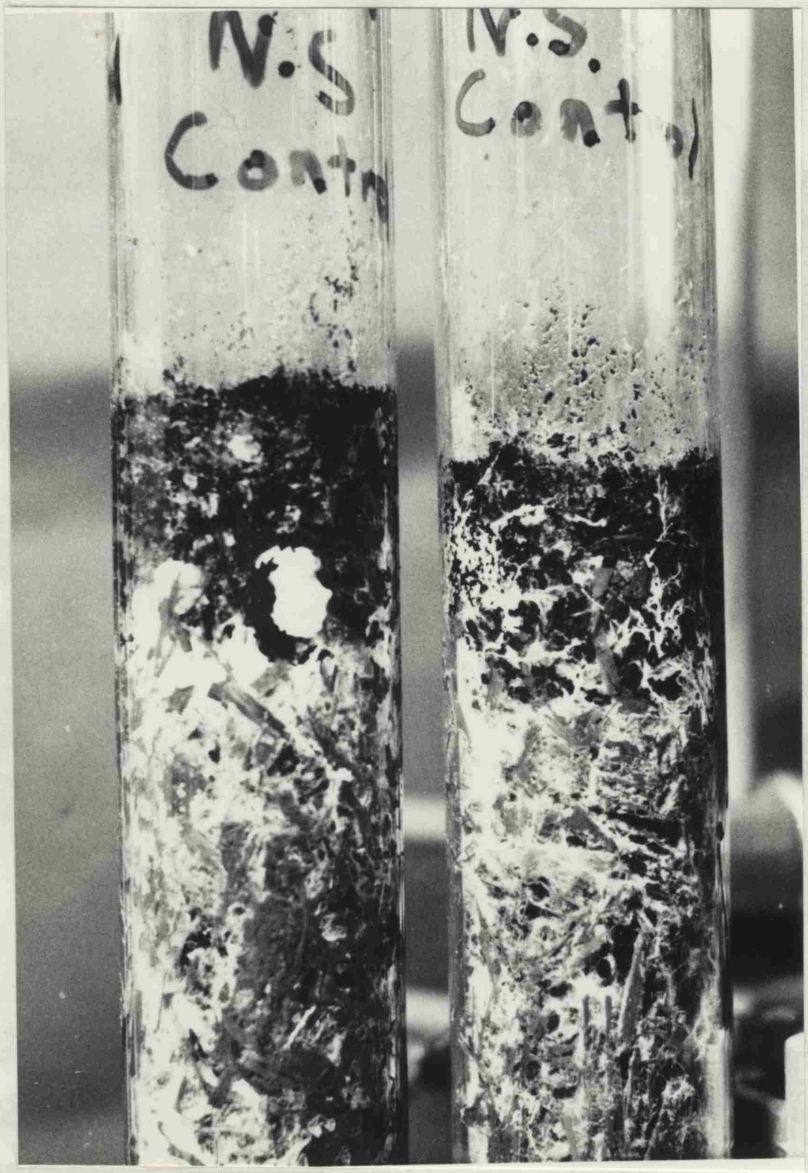

Fig.18. Growth chambers with sterilised compost and unsterilised casing; after 21 days. Sporophore initials and later stages are present .

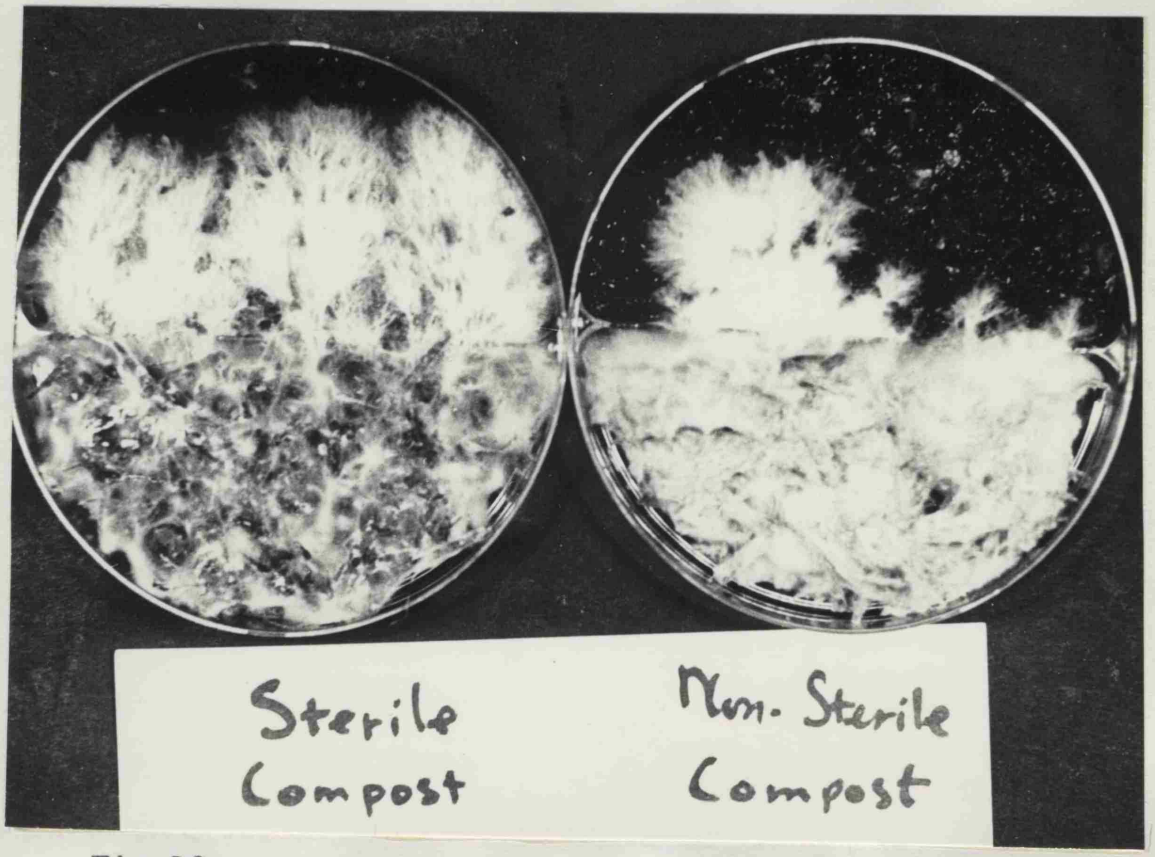

Fig.19. Transversely divided petri-dishes with sterilised casing and ,left, sterilised or, right, unsterilised compost.

would be a necessary prerequisite of microbial transfer in these experiments was demonstrated by direct observation.

Examination of the hyphosphere flora (c.f. Winter, 1960) around the mycelium and of organisms situated upon the hyphal surfaces (the 'hyphoplane') was attempted using modifications of Cholodny's (1930) buried slide technique. Cellulose nitrate coated microscope slides were buried to a predetermined depth in a small box of spawn run compost. A 2 cm. sphagnum peat casing layer was packed around the slides and kept moist by Flegg's (1962) capillary watering system. Three slides were removed at daily intervals and stained with 0.2% cotton blue in lactophenol prior to microscopic examination. Additionally, 23 x 10 x 7.5 cm plastic, lidded, boxes were packed for one third of their length with spawn-run compost and filled with sphagnum peat or University soil. Following strand growth and initial formation, well washed, wet, microscope slides were pressed horizontally on to the casing surface and left in contact for periods ranging from 48 hours to 28 days. Upon removal the slides were stained and examined as previously, except for a few which were Gram stained. In many instances bacteria were seen in close association with hyphal strands (Fig. 20) with their long axes parallel to the hyphal wall. Relatively few organisms were noted in the vicinity of hyphae with densely staining contents (Fig. 21), but they occurred frequently around vacuolated or disintegrating hyphae. It was often difficult to distinguish between small calcium oxalate crystals and bacteria on hyphal surfaces. Fungi other than A. bisporus were not observed, but filaments were seen which may have been actinomycetes.

In order to establish unequivocally the participation of microorganisms in the initiation process pure cultures of isolates from

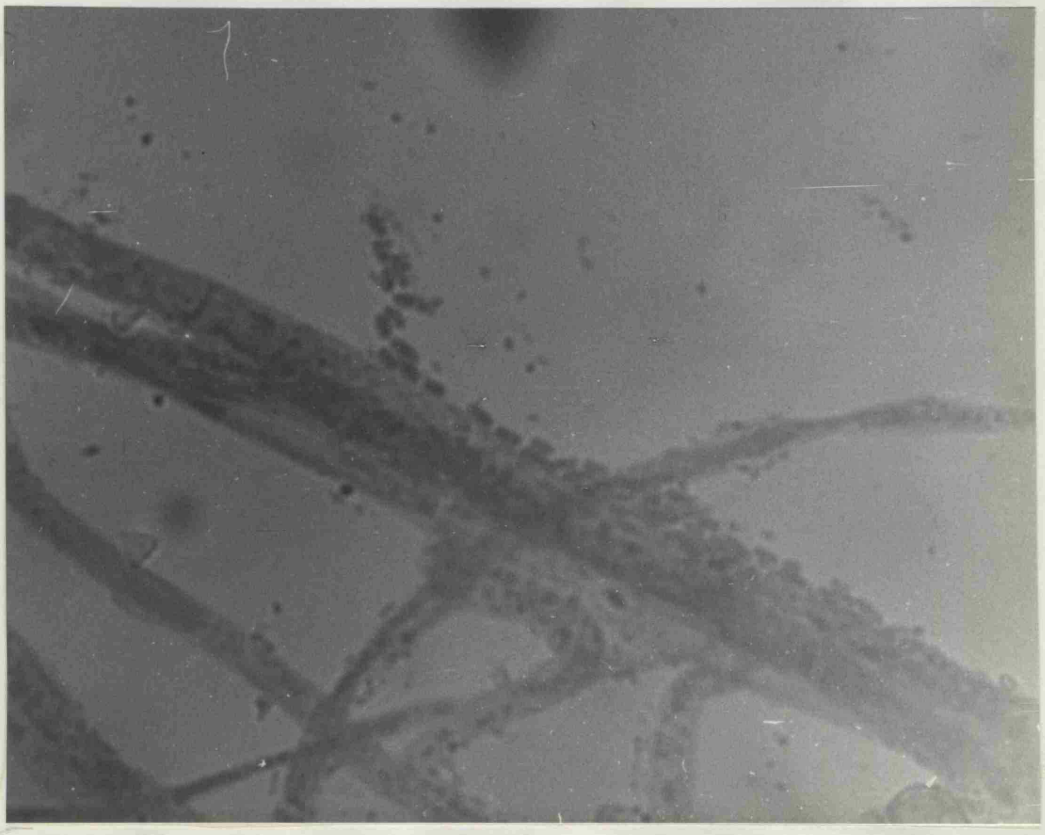

Fig.20. Bacteria associated with mushroom hyphae. (X 1,000) . The slide was in contact with soil casing for 48 hrs. followed by staining in 0.2% Cotton Blue in lactophenol.

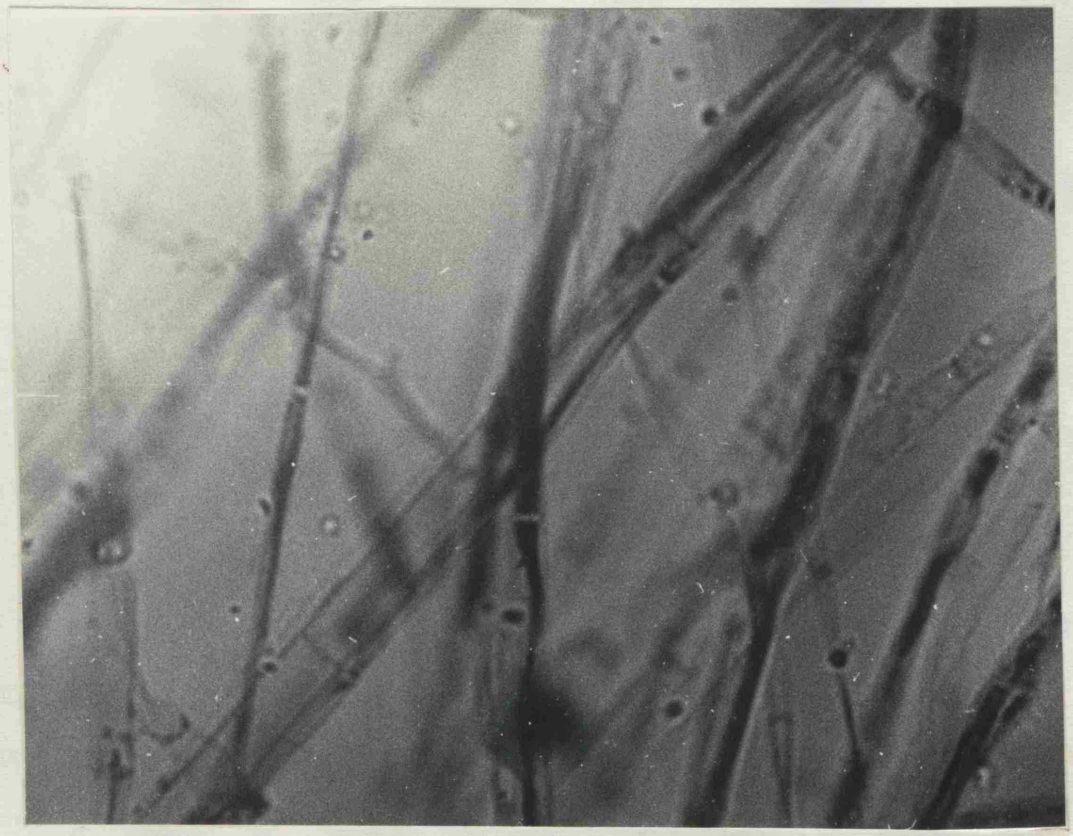

Fig. 21. Dense network of mushroom hyphae with few visible bacteria. (X 1,000) . The slide was treated as in Fig. 20.

casing were tested in sterilised materials. Attention was concentrated upon bacteria as these had been most frequently implicated in fruit body formation. Organisms were isolated and maintained on Nutrient Agar (Oxoid), Topping's medium as modified by Keddie, Leaske and Grainger (1966) and Soil Extract Agar. The latter was prepared by autoclaving 500 g. well colonised peat casing one litre of 0.2% aqueous ammonium phosphate at 121°C for one hour. After paper filtration the volume was restored to one litre, 5 g. yeast extract (Oxoid) and 15 g. agar (Oxoid No. 3) were added and the medium dispersed in 15 ml aliquots prior to re-autoclaving at 121°C for 15 minutes. Organisms were grown at 22° or 25°C.

The first group of bacterial isolates examined was obtained from unused sphagnum peat casing by the pour plate method, using tenfold dilutions prepared from 1 g. material suspended in 10 ml. sterile distilled water. In a rapid preliminary screening in sterilised casing in single half-plates six of the forty four test organisms showed possible activity when added as 2 ml. suspensions taken from 48 hour Nutrient Broth cultures. Two isolates, 1/65 (Bacillus mycoides) and 13/65, a yellow pigmented Gram negative rod, appeared particularly effective, but these and the other isolates showed no activity in further trials. Washed cell suspensions and culture filtrates of 1/65 and 13/65, grown for 72 hours in 50 ml. Nutrient Broth in 250 ml. Erlenmayer flasks on a New Brunswick rotary shaker at 300 r.p.m., were also ineffective. Similar cultures of Uragama's (1960) Bacillus psilocybe also gave negative results.

A further group of sixty four bacterial isolates were obtained from the 'Y' plate experiment which yielded the data quoted in Table 3. These were obtained mainly from peat plus initials in sectors of

Compost	Casing sectors					
	Sterile			Non-sterile		
	Total Sectors	Initials present Nos.	%	Total Sectors	Initials present Nos.	%
Sterile Control	10	0	0	10	8	80
Non-sterile (Farm)	50	17	34	30	30	100

Table 3 Invasion of sterile sectors in Y partioned plates.

sterilised casing colonised from non-sterile compost. It was considered that a selected microflora with an increased proportion of active organisms would be present in this situation. Isolation was achieved by streaking crumbs of peat or dissected initials on Nutrient Agar and Soil Extract Agar. Alternatively single initials or 1 g. peat were shaken thoroughly in 9 ml sterile 0.1% peptone water prior to plating out. After three days incubation isolated colonies were subcultured and stored at room temperature. The isolates were characterised by colony type, cell morphology and Gram reaction. Of the sixty four bacteria obtained in this way 42% were coryneforms, 23% Gram-variable cocci and cocco-bacillary forms, 23% Gram negative rods or cocci and 12% Bacillus spp. Thirty two of these organisms were obtained from sporophore initials and this group included a higher proportion of coryneforms (52%).

The bacteria from initials were screened in triplicate sterile half-plates nine days after isolation by adding 1 ml of a dense suspension in 0.1% peptone water, taken from the original slope separately to the casing and compost. Ten uninoculated controls were included. Six organisms reduced the density of strand growth after 21 days in the casing, compared with the controls, while two, designated 45/67 and 67/67 and both yellow coryneforms, induced single initials without visibly affecting hyphal development. These eight isolates were re-screened in half-plates containing sterilised materials with the remaining thirty two organisms from peat and fourteen other yellow pigmented coryneforms obtained previously from casing on commercial mushroom beds. The latter were isolated by the pour plate technique using Topping's medium or Soil Extract Agar and stored on slopes of these media prior to examination. The screening procedure was simi-

lar but four day Nutrient Agar cultures were employed. Ten replicates of isolates 45/67 and 67/67 were included, and all other organism tested in triplicate. None of the previously active organisms induced sporphore formation after 21 days although 45/67 consistently reduced the density of strand growth. A single initial occurred in two plates inoculated with 42/67 and 53/67 respectively.

Only eighteen of the fifty-four test organisms were recovered at the termination of this experiment. Consequently cultures for the final sterile half-plate screening were pre-incubated in the casing. The organisms examined were seventeen isolates obtained from a single 'Y' plate containing initials, and included 45/67. Air dried sphagnum peat was saturated with an aqueous solution of 1% glycerol (B.D.H.), 0.5% dried yeast extract (Oxoid) and 0.5% casein hydrolysate (Oxoid), distributed in petri-dishes (50 g.) and autoclaved for thirty minutes at 121°C on three successive days. After inoculation with 1 ml. 0.1% peptone water suspensions of the test organisms the peat was incubated for 5 days before being used as casing in ten replicate half-plates for each isolate. Sampling on Nutrient Agar indicated that all isolates were established in pure culture at the time of casing. Twenty-one days later none of the test organisms had induced initiation and density of stranding was reduced only in the presence of a mixed population of all isolates.

Coincident with the second screening of 'Y' plate isolates the coryneform 45/67 was also examined in two sterile culture chambers. The casing and the top of the compost were inoculated with 3 ml. of a dense bacterial suspension in 0.1% peptone water, taken from the original slope. Twenty one days later seven sporophore initials had

formed in one chamber and strand growth was retarded in both. Colonies with the characteristic appearance of 45/67 were recovered from the casing when plated on to Nutrient Agar. The same organism was included in a second culture chamber experiment with another seven isolates that had either induced initiation or restricted strand development. All these organisms were pre-incubated in nutritionally enriched casing, but none showed any activity against growth and development of hyphal strands.

Because of the difficulties encountered in re-establishing bacteria in casing and the apparent rapid loss of activity exemplified by 45/67, no further attempts were made to isolate specific active micro-organisms.

In an attempts to confirm the microbial role in sporophore initiation, 1 ml. doses of antibacterial antibiotics were added to non-sterile casing in the hope that they would suppress carpogenesis. Those examined were streptomycin sulphate (Glaxo), 1490 and 149 units per ml.; benzyl penicillin (Glaxo) 2000 and 200 units per ml., and neomycin sulphate (UpJohn) 100 µg per ml. Streptomycin and neomycin were included as broad spectrum antibiotics, the latter being active against pseudomonad bacteria. Penicillin and streptomycin were added in 0.2 M phosphate buffer, pH 7.0. Relatively high doses were utilised on account of the instability of antibiotics in soil.

Only neomycin suppressed initiation (Table 4; Fig. 22) a result not confirmed in two subsequent trials. With penicillin and streptomycin initials formed principally around the addition site (Fig. 23) where strand density was reduced. On re-testing it was found that this localised reaction was induced by the phosphate buffer, added to

Antibiotic	Dose per ml.	% Plates with Initials	
		7 days	14 days
Control	0	20	60
Penicillin	200 I.U.	40	60
Penicillin	2000 I.U.	30	80
Streptomycin	149 I.U.	0	10
Streptomycin	1490 I.U.	40	50
Neomycin sulphate	100 µg.	0	0

Table 4. Effect of Antibacterial Antibiotics in Non-Sterile Half-plates.

Fig.22. The effect of adding 100μg neomycin centrally to unsterilised casing.

Fig.23. The effects of adding 200 I.U.'s penicillin in buffer centrally to unsterilised casing. (at 14 days)

all treated plates, 60% of which produced initials compared with 40% of the untreated controls. Neither buffer nor antibiotics had any effects in sterile half-plates. To determine which were the active components of the buffer system equimolar solutions of NaH_2PO_4, Na_2HPO_4, NaCl and the equivalent potassium salts were tested separately. Tris-maleate buffer pH 7.0, was included as a check on possible pH effects. The results are presented in Table 5 and suggest that the active fraction was the metaphosphate moiety, although the effect was much more pronounced with the buffer than with the single salt solutions.

One interpretation of the failure to obtain sporophores in autoclaved materials is the production of toxic substances during sterilisation (Tschierpe and Sinden 1964). No evidence has been produced to support this concept. Increases in solutes, including free hexose, which are known to occur in autoclaved soils (Peterson, 1962· Ko and Lockwood, 1967), might also modify the behaviour of hyphae in sterilised casing. The following approaches were adopted to avoid or remove materials from autoclaved casing.

i) University soil and sphagnum peat were sterilised with propylene oxide.

ii) Sphagnum peat (200 g.) was washed with running tap water for 24 hours prior to autoclaving.

iii) Aliquots of sphagnum peat (25 g.) were autoclaved at 121°C for 30 minutes on three successive days in 250 ml. polypropylene centrifuge bottles and then washed with four changes of 100 ml. sterile distilled water by centrifugation at 4,000 x g. for 15 minutes before use.

Additive 1 ml of 0.2M solutions	% Plates at 7 days with	
	Initials + Inhibition	Inhibition only
Control H_2O added	0	30
Phosphate Buffer	50	40
Tris maleate Buffer	20	10
NaH_2PO_4	0	40
Na_2HPO_4	0	60
NaCL	10 (no inhibition)	0
KH_2PO_4	10	30
K_2HPO_4	10	20
KCl	0	0

Table 5. The effects of 0.2M Phosphate Buffer and its component salts in non-sterile half-plates

iv) Autoclaved sphagnum peat (80 g.) was packed aseptically into a pre-sterilised 3.6 x 27.0 cm. glass tube fitted with perforated rubber bungs to permit perfusion with sterile water. The apparatus was connected by sterile latex tubing to a Whatman Gamma 10 resin-bonded fibre bacteriological filter. Tap water was then passed through the apparatus at 300 ml./minute for 18 hours prior to use.

None of the treatments led to sporophore production in culture chambers containing sterile materials and did not affect hyphal growth.

The amount of free hexose was determined in membrane filtered 1 ml aliquots of 100 ml. aqueous extracts of 25 g. samples of peat casing treated as follows: untreated; autoclaved; autoclaved and washed; or autoclaved and inoculated with 0.5 g. non-sterile casing. The latter sample was incubated, after thoroughly mixing the inoculum at 50°C for 36 hours to encourage growth of thermophiles, which it was hoped would reduce the nutrient levels. Hexose was estimated as glucose equivalents by the phenol-sulphuric acid method of Dubois et al (1951). The initial free hexose content of the samples and mycelial growth rates in similarly treated casing are shown in Table 6. In spite of a marked increase in free sugars in autoclaved casing the growth rate decreased only slightly when free hexose was reduced by washing to levels found in untreated peat. Initials occurred only in unsterilised casing, while growth in the other cases continued uninterrupted. Thus strand growth was hardly affected by a low level of nutrients in sterile casing. Although leaching of possibly toxic substances could not be demonstrated, this could have occurred during the washing procedure. Since propylene oxide treatment was

Casing treatment	Free hexose[1] µg/g dry casing.	Average daily hyphal growth mm[2]
Autoclaved	3,160	1.3
Autoclaved + thermophiles	1,590	1.3
Autoclaved washed	150	1.1
Untreated control	176	1.0

1. As glucose equivalents.

2. As measured over 14 days.

Table 6. The effect of nutrient status on hyphal growth into casing.

equally effective in suppressing initiation and promoting hyphal growth it is unlikely that toxic materials are produced during autoclaving of casing or compost materials. It is also significant that inoculation of propylene oxide sterilised peat with non-sterile soil led to sporophore initiation.

Summary

The experimental data presented in this section, while failing to demonstrate unequivocally that microbes participate in the initiation process nevertheless supports the observations of Eger (1959 - 1965b) and Thomas et al (1964). Bacterial isolates failed to induce initiation when added singly to casing, but bacteria were observed in close association with mushroom hyphae. The nutrient status of casing, using free hexose as an index, was increased by autoclaving but this, in itself, had little effect on the pattern and rate of mycelial growth. The formation of heat induced toxins appears unlikely, since gaseous sterilisation has similar effects to autoclaving on hyphal growth. while the effect of addition of unsterile soil or peat sterilised casing is also difficult to explain in this context.

3. Volatiles other than CO_2

Although the production of sporophores in downward ventilated, culture chambers appeared to eliminate the involvement of such materials in sporphore initiation, some consideration was given to their possible activity in aseptic culture as suggested by Schisler (1957), Lockard (1962) and Lockard and Kneebone (1962), using large ventilated Erlenmeyer flasks. These workers emphasised that air had to be drawn through the flasks with the orifice of the air outlet tube as close as possible to the casing surface and with the inlet tube just perforating the flask stopper. The need for this arrangement is explicable either through the efficient removal of excess CO_2 from the casing surface or by an enhanced movement of Schisler's volatile "hormone" into the casing.

Three experiments were performed to test the latter possibility. In these the air flow to the growth chambers was modified (Fig. 24) so that sterile chambers were ventilated with air that had first passed through another culture which acted as a source for possible volatiles. Culture chambers containing sterile or non-sterile compost, developing sporophores or 1 litre Erlenmeter flasks with 300 g. spawn-run compost were used for the latter purpose. Carbon dioxide absorbent columns were placed between the two cultures and an air bleed was inserted between the absorbent and the filter to restore CO_2 levels to those permitting carpogenesis. Considerable problems were encountered with this more complex flow-line, particularly drying out of the aseptic cultures due to development of negative pressures. This difficulty was not completely overcome. No clearly defined sporophores developed up to 21 days after casing in any of the treatments.

Concurrent with these experiments the possible activity of ethylene was examined. This gas was selected for study on account of its

Casing treatment	Free hexose[1] µg/g dry casing.	Average daily hyphal growth mm[2]
Autoclaved	3,160	1.3
Autoclaved + thermophiles	1,590	1.3
Autoclaved washed	150	1.1
Untreated control	176	1.0

1. As glucose equivalents.

2. As measured over 14 days.

Table 6. The effect of nutrient status on hyphal growth into casing.

equally effective in suppressing initiation and promoting hyphal growth it is unlikely that toxic materials are produced during autoclaving of casing or compost materials. It is also significant that inoculation of propylene oxide sterilised peat with non-sterile soil led to sporophore initiation.

Summary

The experimental data presented in this section, while failing to demonstrate unequivocally that microbes participate in the initiation process nevertheless supports the observations of Eger (1959 - 1965b) and Thomas et al (1964). Bacterial isolates failed to induce initiation when added singly to casing, but bacteria were observed in close association with mushroom hyphae. The nutrient status of casing, using free hexose as an index, was increased by autoclaving but this, in itself, had little effect on the pattern and rate of mycelial growth. The formation of heat induced toxins appears unlikely, since gaseous sterilisation has similar effects to autoclaving on hyphal growth. while the effect of addition of unsterile soil or peat sterilised casing is also difficult to explain in this context.

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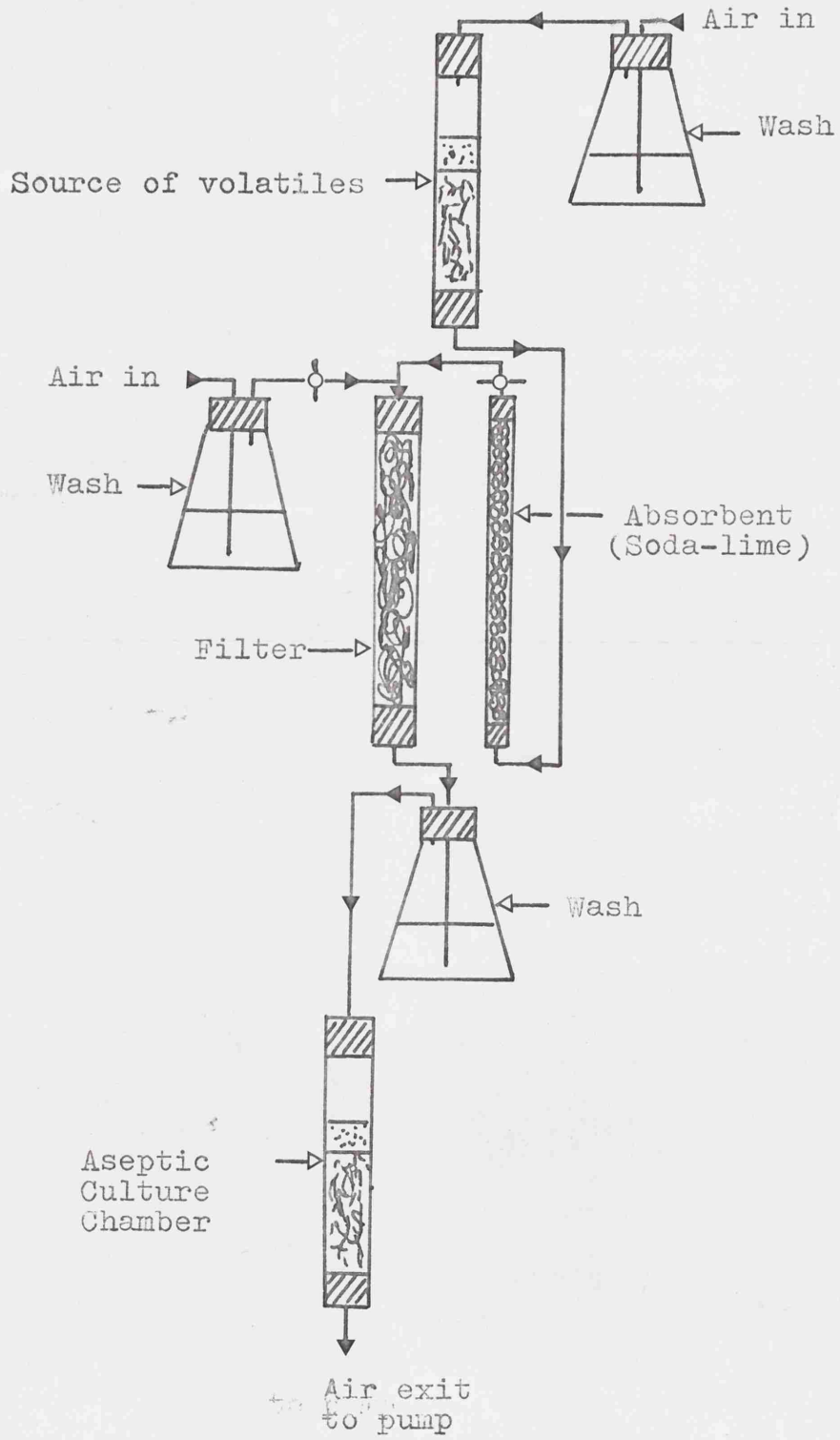

Fig.24. Air flow for testing for volatiles

reported occurrence in mushroom cultures (Lockard 1962, Lockard and Kneebone, 1962) and its known biological activities in trace amounts (Biale, 1960), some of which are counteracted by CO_2 (Smith and Parker, 1966). Air mixtures (British Oxygen Co. Ltd.) containing 0.0; 0.018; 0.086 and 0.95 p.p.m. ethylene and approximately 300 p.p.m. CO_2 were metered through duplicate sterile culture chambers for up to 28 days without effect. Growth curves for the first 14 days illustrated in Fig. 25, show no sign of the check in growth usually associated with sporophore initiation.

Summary

Two attempts were made to obtain direct evidence for participation by volatiles other than CO_2 in the initiation process in A. bisporus. The first approach employed developing cultures of the fungus as a volatile source: the second involved use of ethylene/air mixtures. In neither case were sporophores produced which suggests that volatile metabolites, other than CO_2, produced by the mushroom mycelium are unlikely to be involved in sporophore initiation.

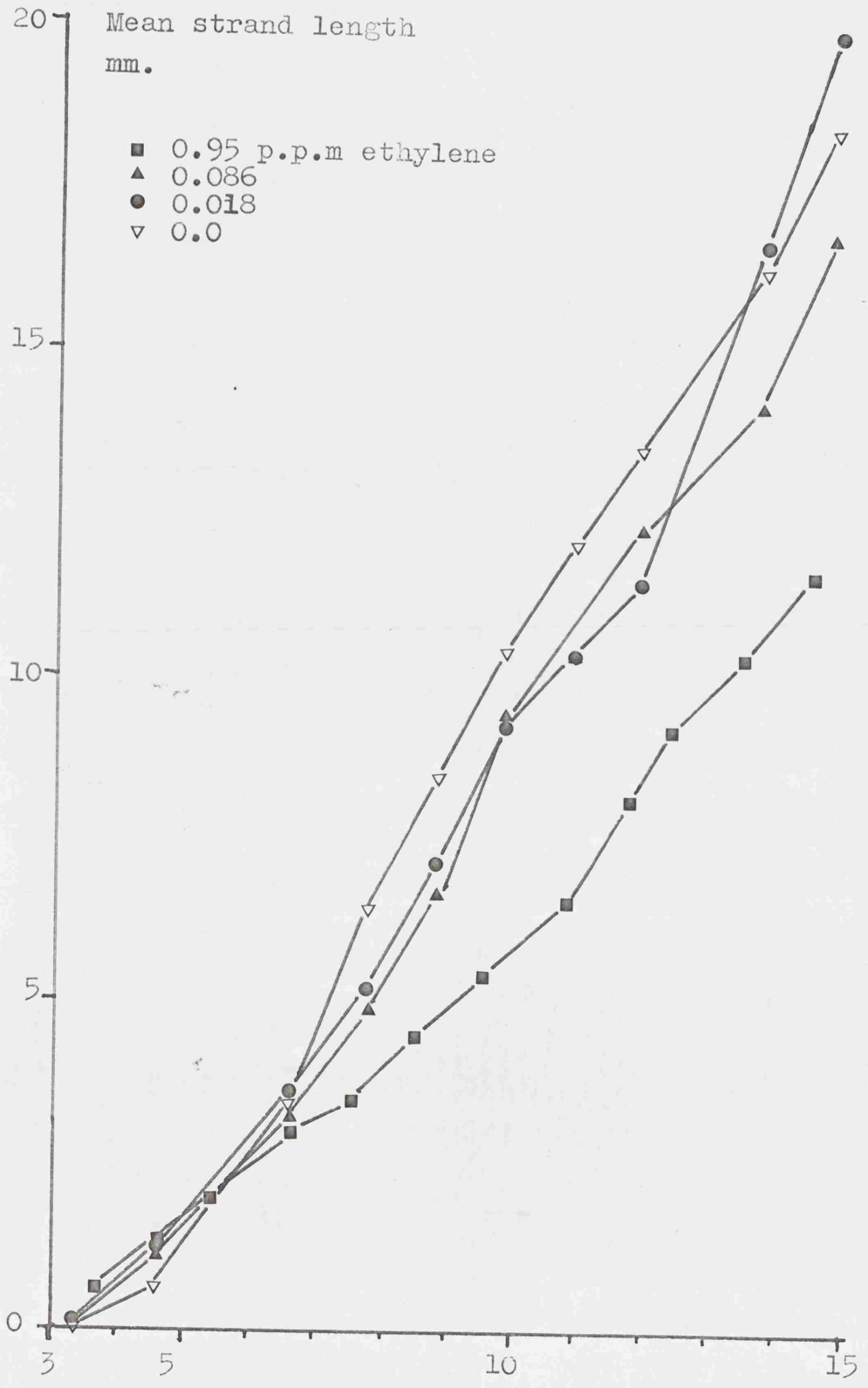

Fig. 25. Strand growth into sterile casing at 0.0 ; 0.018; 0.086 and 0.95 p.p.m. ethylene and approximately 300 p.p.m carbon dioxide.

4. The Role of Micro-organisms

Possible explanations of the role of micro-organisms in promoting carpogenesis in A. bisporus could include:

i) Intervention by exogenous metabolites ('exocrines', Lucas, 1949) or cellular components released on death of the microflora.

ii) Competition for available nutrients rendering the casing nutritionally deficient (c.f. Lambert, 1938; Edwards, 1949).

iii) Removal or modification of metabolites or structural components from the hyphal surfaces and their vicinity, as proposed by Eger (1961).

Investigations in this section can be conveniently considered under the above groupings.

a) Microbial metabolites.

Treschow (1944) and Fraser (1956) have reported that thiamin and biotin were necessary factors for mycelial growth. One ml. doses of biotin (0.1 µg/ml), thiamin, ascorbic acid, nicotinic acid, riboflavine, pyridoxine hydrochloride (all 10 µg/ml), and dried yeast extract (Difco; 50 µg/ml) added to sterile casing half-plates did not promote initiation or affect hyphal growth. Similar results were obtained with larger doses of thiamin (100 µg/ml), biotin (1 µg/ml) and Difco yeast extract (500 µg/ml) added to sterile casing in culture chambers.

Indole-acetic acid stimulates the surface growth of mushroom mycelium in liquid culture (Fraser, 1956). This substance has been reported in quantity from mushroom sporophore tissues by Konishi and Hagimoto (1961) but does not appear to function as a growth hormone here. In sterile casing, incubated in the dark, 1 ml doses of 0.1, 1.0 and 10.0 µg/ml indolyl-3-acetic (Koch-Light) produced no initiation response, or growth check, up to 21 days from casing.

Lipids, possibly sterols, have been suggested as participants in sporophore initiation (Schisler and Sinden, 1966; Schisler, 1967). Coryneform bacteria, a group common in casing soil, although unable to synthesise sterols are capable of transforming them. Lipids, including long chain, branched, unsaturated fatty acids are prominent components of bacterial cell walls and membranes (Rogers and Perkins, 1968). Short chain fatty acids are common end-products of bacterial growth on carbohydrates, and have been implicated as stimulants of mushroom basidiospore germination when present in low concentration (Lösel, 1967).

The following vegetable oils, including most employed by Schisler and Sinden (1966), were examined in sterile casing in half-plates, castor, corn, groundnut, soya and cotton-seed oil. These were sterilised in 10 ml aliquots with 2 ml distilled water by autoclaving at 115°C for 10 minutes, and added in 0.1 ml amounts to the centre of sphagnum peat casing. Additionally, 0.1 ml and 1.0 ml of castor oil was added to the compost in a separate group of plates. All treatments were without effect on hyphal growth and development and failed to induce carpogenesis.

As mushroom tissues contain considerable amounts of linoleic acid (Hughes, 1962) and since unsaturated fatty acids are prominent components of vegetable oils, technical linoleic and oleic acids (B.D.H.) and sodium ricinoleate were included in a screening experiment employing half-plates with the long chain, saturated, palmitic, stearic and myristic acids (B.D.H.). These substances were mixed into dried sphagnum peat at 10 mg. per g. dry weight, prior to wetting and autoclaving. Shorter chain fatty acids were also tested, viz., acetate,

butyrate, crotonate and methyl-aceto-acetate (Sigma). These were sterilised separately as 0.01 M aqueous solutions by autoclaving at 110°C for ten minutes, and added in 1 ml aliquots to the casing. None of the long or short chain fatty acids induced initiation, although the latter and sodium ricinuleate inhibited growth of strands into the casing around the application site. Sterols were also examined in view of their role in morphogenesis in Pythiaceous fungi (Elliot, Hendrie, Knights and Parker, 1964; Hendrix, 1964; Klemmer and Lenney, 1964). Cholesterol and two plant sterols, stigmasterol and β-sitosterol (all supplied by Sigma) were included in the study. When added at 10 μg per g. dry casing prior to wetting and sterilisation, none of these substances had any effect.

The results of additions of vegetable oils and unsaturated fatty acids to unsterilised casing and compost in half-plates are summarised in Table 7 and 8. The numbers of initials produced were inconsistent. Groundnut oil, apparently stimulated initiation in three of six experiments. In one experiment, where initials were numerous in the controls, groundnut oil added as an emulsion to compost increased the numbers of sporophores at the lowest concentrations, but had no effect in casing where fruit body numbers declined markedly with increasing dosages. A parallel but less marked response occurred with linoleic acid in casing. When added to the centre of the casing layer 0.1 ml sodium ricinoleate or glycerol tributyrate retarded hyphal development in the immediate vicinity, but did not promote initiation. Other lipid materials produced no localised effects, but the short chain fatty acids examined earlier in sterile casing were also inhibitory to mycelial growth in non-sterile materials.

Oil or fatty acid	Dose ml.	Method Added	Mean number of initials per plate.				
			Added to casing			Added to compost	
			4/67	6/67	15/67	4/67	15/67
None, control			6.4	2.0	10.3	6.4	10.3
Groundnut	0.1	Centrally	17.2	8.1			
"	0.1	Emulsion[1]			5.5		
"	0.5	" "			2.3		
"	1.0	" "			3.5		7.6
"	2.5	" "			2.3		10.2
"	5.0	" "			2.2		8.7
Castor	0.1	Centrally	16.9	8.2		7.3	
	1.0	"	6.2			7.9	
Cotton seed	0.1	"	11.4	5.4		6.1	
*Corn	0.1	"	12.2	17.2			
Soya	0.1	"	9.3	6.9			
Linoleic acid	1.0	Emulsion[1]			8.2		
" "	2.5	"			0.7		
" "	5.0	"			1.3		
Na ricinoleate	0.1	Centrally			7.1		
Experimental Duration (Days)			14	14	21	14	21

* Dose 1.0 ml. to casing omitted - heavy contamination.

[1] Doses added to 100 g. dry casing or 100 g. fresh compost.

Table 7. Addition of Vegetable oils and Unsaturated Fatty acids to non-sterile half-plates. Sphagnum peat casing.

Oil or Fatty acid	Dose ml.	Method Added	Mean numbers initials per plate			
			Added to casing			Added to compost
			11/67	13/67	14/67	14/67
None, control	0		29.8	24.5	29.8	29.8
Groundnut	0.1	Centrally	52.6			
"	0.1	Emulsion[1]		12.5		
"	1.0	"		1.7	30.6	49.7
"	2.5	"			6.0	48.7
"	5.0	"			2.2	20.5
Castor oil	0.1	Centrally	23.5			
Cotton seed	0.1	"	34.2			
Corn	0.1	"	26.3			
Soya	0.1	"	28.6			
Glycerol tributyrate	0.1	"	25.1			
Linoleic acid	0.1	"	40.7	33.3		
Oleic acid	0.1	"	35.2	4.0		
Sodium ricinoleate	0.1	"			22.3	
Experimental Duration, (Days)			21	16	21	21

[1]Doses added to 100 g. dry casing or 100 g. fresh compost.

Table 8. Addition of Vegetable oils and Unsaturated Fatty acids to Non-sterile Half-plates. Somerset Sedge Peat casing.

Linoleic and oleic acid, added locally, had no marked effects on initiation.

When using Somerset sedge peat a high incidence of fungal contamination was observed upon introducing spot additions of crude lipid sources and unsaturated long chain fatty acid, (Table 9). The most prominent organism was Ostracoderma sp. which sporulated over the entire casing surface. The effect was not noted when lipids were added as an emulsion throughout the casing.

In Hendrix and Lauder's (1966) study on Pythium periplocum the inhibitory effects of polyene antibiotics on oospore formation were overcome by sterol supplementation, demonstrating that the action mechanism for these antibiotics included interference with specific steroid function, as suggested for Saccharomyces cerevisiae by Gottlieb et al (1961). If polyene antibiotics selectively interfered with or induced initiation in the cultivated mushroom this would provide some indication of steroid involvement in the morphogenesis. Accordingly, samples of most of the polyene antibiotics utilised by Hendrix and Lauder were screened in half-plate cultures. Hyphal growth into sterile casing was retarded in the presence of 1 ml doses containing 300 µg filipin (aqueous solution; 52% pure; UpJohn) or 150 µg pimaricin (Cynamid; in 5% dimethyl sulphoxide) but no initials were produced. Similar retardation of hyphal growth occurred with these concentrations in non-sterile casing, and with aqueous solutions 1 ml of 260 µg/ml mycostatin (Squibb) or 275 µg/ml amphotericin β (Squibb). Table 10 contains the results of a half-plate experiment employing 1 ml doses 1.5, 25 and 250 µg/ml aqueous pimaricin (Royal Dutch Fermentation Industries) or 2.7, 26.5 and 265 µg/ml filipin (95% pure, UpJohn) in

Oil or fatty acid	11/67		13/67		14/67	
	Contaminated plates	% with Ostracoderma	Contaminated plates	% with Ostracoderma	Contaminated plates	% with Ostracoderma
Groundnut	10	70				
Castor	10	100				
Cotton	10	30				
Corn	9	22				
Soya	7	14				
Glycerol tributyrate	10	0				
Linoleic acid	8	50	8	100		
Oleic acid	10	60	7	100		
Sodium ricinoleate					10	100

Table 9. Occurrence of 'sporulating 'Ostracoderma' sp. in Oil Supplemented Somerset Sedge Peat. Dose 0.1 ml added Centrally.

Antibiotic	Dose µg per ml.	Mean Initials per plate at 16 days	% plates with checked hyphal growth	
			8 days	16 days
Control, None	0	24.5	0	0
Pimaricin (aqueous)	2.5	10.7	0	0
"	2.5	12.9	0	0
"	250	18.6	80	30
Filipin (in 5% DMSO)	2.7	16.5	0	0
"	26.5	31.7	40	0
"	265	4.6	100	60

Table 10. The Effects of Polyene antibiotics in Non-Sterile Half-plates. Expt. 13/67

5% dimethyl sulphoxide, added locally to non-sterile sphagnum peat casing. Hyphal growth was inhibited only at the highest levels of both antibiotics but the numbers of initials were reduced except with filipin (25 µg/ml). This suggests a difference in sensitivity to the antibiotic for vegetative and reproductive growth. Two attempts were made to overcome inhibition of growth by pimaricin with increasing doses of cholesterol. According to Hendrix and Lauder (1966) a 1 : 10 ratio of sterol to antibiotic was sufficient to counteract any inhibitory effects in Pythium periplocum. Steroid : polyene ratios from 1: 100 to 10 : 1 were examined as follows. A sterile aqueous suspension of pimaricin, as used in the previous experiment, was incorporated in 100 ml 2% Malt Extract Agar at 25 µg/ml and 250 µg/ml. Cholesterol (Sigma) was added in glycerol trioleate (B.D.H.) at 2.5, 25, 125 and 250 µg/ml. The final triolein concentration was 1 ml per 100 ml medium. Antibiotic, steroid and triolein free controls were included. Five plates were poured from each 100 ml medium and were inoculated with a 5 mm. disc of mycelium from the periphery of a 14 day old colony on 2% Malt Extract Agar. The plates were incubated in the dark at 25°C for 21 days. Inhibition of growth was complete at both pimaricin levels and was not overcome by any concentration of cholesterol. There was no evidence for increased growth in response to steroid in polyene free controls. These findings might be explained by the failure to incorporate or utilise steroid in the form supplied.

One property of lipid membranes is their ability to participate in ion exchanges (Kimizuka, Nakhara, Uejo and Yamauchi, 1967) involving Ca^{++}, Na^{+} and K^{+}. Uptake of Ca^{++} is of particular significance as this ion is required by the mushroom (Treschow, 1944) and is deposited

as calcium oxalate crystals on mycelium and initials (Eger and Sücker, 1964). In an attempt to interfere with ion exchanges at hyphal surfaces, the chelating agent ethane-daimine-tetra-acetic acid (i.e. E.D.T.A.) was added, as the disodium salt in aqueous solution to the casing of half-plates. No effects were observed following addition of separately sterilised 0.1M E.D.T.A. to sterile cultures, although when autoclaved in the peat it proved toxic. In non-sterile casing (Table 11) numbers of initials were directly proportional to concentration, marked increases being obtained with 0.1M E.D.T.A.

A heterogenous group of substances was added to sterile or non-sterile half-plates in empirical attempts to simulate microbial action. These included the antibiotic griseofulvin: cysteine, to provide lowered redox potentials, and sulphanilamide which can retard active uptake of CO_2 by inhibition of carbonic anhydrase systems. All were without effect upon initiation, although griseofulvin (150 µg) inhibited hyphal development.

b) Nutritional deficiency.

One of the characteristics of mycelial growth in casing, associated with production of sporophore initials, was the relatively sparse network of thick hyphal strands (c.f. Fig. 1 or 15). Matthew (1961) has demonstrated in sand culture that these were developed in response to low nutrient status of the medium.

All attempts to promote sporophore initiation by exposure of growing hyphae to a nutrient deficit failed. Hyphal growth was sparse on acid-washed Diatomite (Moler Products Ltd.) or quartz sand in sterile partioned and normal half-plates, but no initials formed.

Conc^{n} E.D.T.A.	Sterili-sation	Mean numbers of initials per plate.			
		11/67[1]	13/67[2]	14/67[2]	75/67[2,3]
None, Control		29.8	24.5	29.8	10.2
0.001M	Untreated		3.3	38.6	16.2
0.01M	"		14.8	58.4	6.9
0.1M	"	59.0	19.4	57.0	38.3
0.001M	Autoclaved			29.8	12.4
0.01M	"			42.6	20.4
0.1M	"			50.8	46.2
Duration (Days)		21	16	21	21

1. Added as 1 ml to the centre of the casing.

2. Added to dry casing at 150 ml per 100g dry casing.

3. Sphagnum peat.

Table 11. Effect of a Chelating Agent, Ethane-diamino-tetracetic acid (E.D.T.A.) in non-sterile half-plates.

Somerset sedge peat casing.

Similar findings were obtained when water agar was substituted for casing in transversely divided plates. In one of these plates an aberrant pinhead stage sporophore formed adjacent to the partition, in close proximity to a submerged colony of a fungal contaminant, Cladosporium sp. An aseptic culture chamber experiment was also performed with sterile and non-sterile "Diatomite" as a casing. The "Diatomite" was separated from the compost by a 5 mm gap formed by insertion of a polyvinylchloride foil partition, in order to reduce diffusion of solutes into the casing layer. A second partition was inserted 1 cm from the base of the casing. The experiment terminated after fifteen days. Initials had formed in the non-sterile peat controls, but not in sterile or non-sterile "Diatomite" although this was colonised by mycelium.

Nutritional supplementation of the casing offered an alternative approach to assessment of the Klebsian hypothesis as interpreted by Lambert, 1938 and Edward, 1949. Flegg (1956, 1957) had reported that addition of nutrients to the casing tended to reduce sporophore yields; which would be expected were fruiting a response to limited nutrition. Half-plate experiments provided further evidence on this point. Aliquots (1 ml) of 0.15, 1.5 and 15% glucose, 1% and 10% casein hydrolysate (Oxoid) and Nutrient Broth (Oxoid) were pipetted on to unsterilised casing 4 days after filling the plates. Fourteen days later initials had developed in 90% of the controls, 30% of the plates with 1.5% glucose and 20% of the plates with 1% casein hydrolysate, and in none of the other treatments. The highest concentrations of glucose and casein hydrolysate were strongly inhibitory to mycelial growth (e.g. Fig. 26), the remaining supplemented plates showed dense,

Fig.26. The effect of adding 15% glucose (1 ml.) centrally to unsterilised casing. (at 14 days)

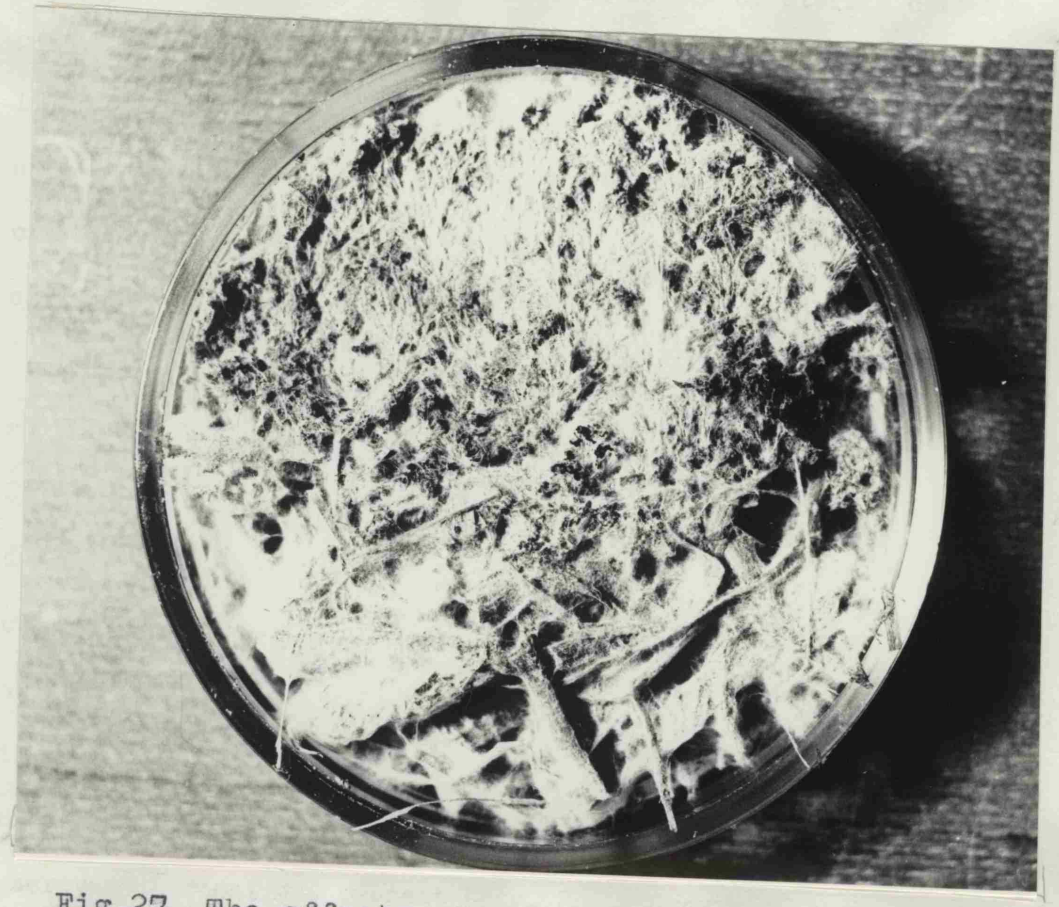

Fig.27. The effects of adding 0.15% glucose (1 ml.) centrally to unsterilised casing. (at 14 days)

fine strand growth (Fig. 27). A further experiment indicating the effect of adding increased carbon source has been described previously (page 37) where increasing the groundnut oil content of casing greatly decreased numbers of initials while not restricting hyphal growth.

c) Removal of hyphal metabolites.

Eger (1961) recorded initiation in 60% of sterile cultures cased with activated charcoal, although numbers of sporophores were low. The incidence of sporophore initiation increased when charcoal and soil were mixed, probably because of improved water holding capacity. No explanation could be provided for the stimulation of carpogenesis by the charcoal.

An early attempt to repeat these observations employing gas adsorbent charcoal (B.D.H.) failed. Following discussion with Dr. Eger the procedure was repeated and extended using transversely divided petridishes and sterilised materials. The peat/charcoal casing employed contained the following percentages of charcoal on a dry weight basis: 0, 25, 50, 75 and 100. The results are summarised in Table 12 and have been confirmed in a later experiment with undivided plates. Initials formed in a proportion of the plates wherever charcoal was present (Fig. 28). Growth of hyphae across the partitions was strikingly retarded by the presence of charcoal, where it did occur stranding was similar to that in non-sterile peat casing (Fig. 29). In a repeat of this experiment very few initials formed because of poor colonisation of the casing. Two attempts were made to simulate the action of charcoal by use of other adsorbent or surface active materials. With transversely divided petri-dishes

Casing % Charcoal weight/ weight	Time after casing.						
	21 days			28 days		35 days	
	Max. strand length (mean) m.m.	Initials		Initials		Initials	
		F%	$\bar{\mu}$	F%	$\bar{\mu}$	F%	$\bar{\mu}$
Control 0% Peat only	34.6	0	0	0	0	0	0
25%	13.9	50	1.8	50	2.1	50	4.3
50%	3.4	30	0.4	40	0.9	40	2.1
75%	4.3	30	5.6	50	12.1	60	15.8
100%	2.0	30	2.0	40	5.1	50	6.2

F% = Percentage plates with initials.

$\bar{\mu}$ = Mean numbers per plate

Table 12. Effect of Gas-adsorbent Charcoal in sterile casing.

Transversely partioned Half-plates.

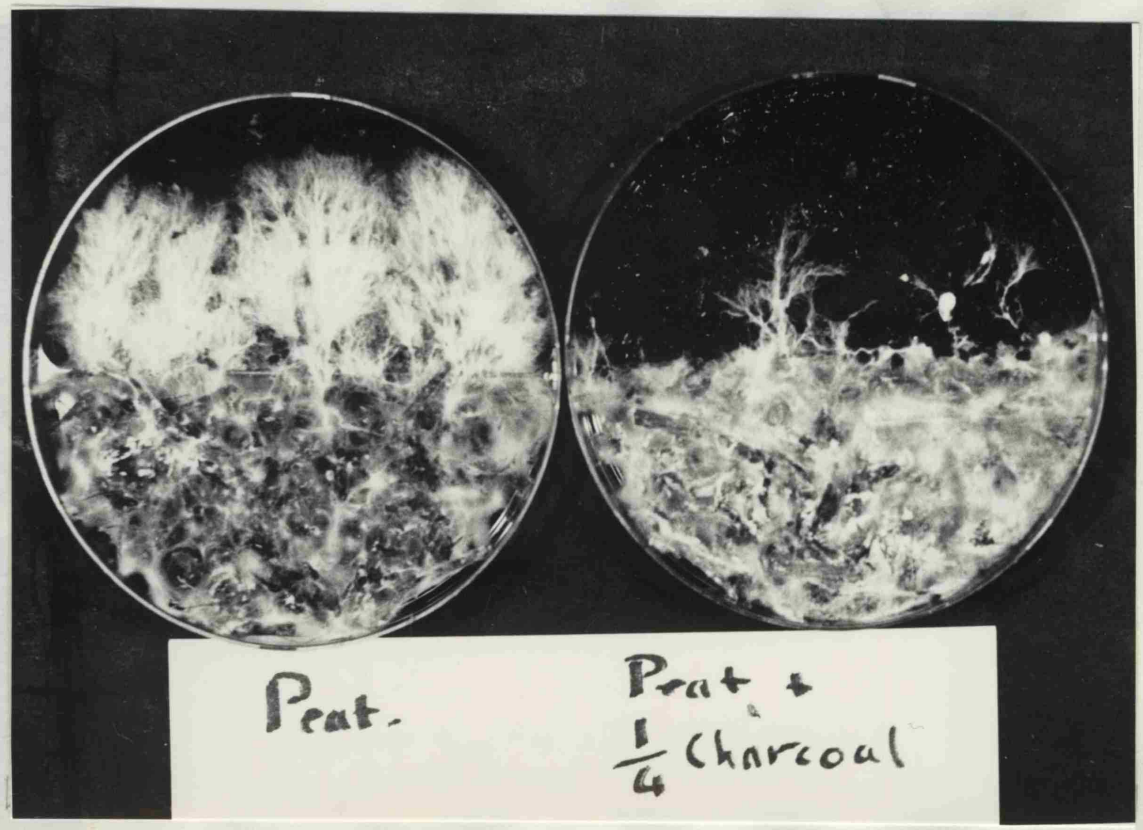

Fig. 28. The contrast in growth of pure cultures on autoclaved sphagnum peat (left) and peat with 25% (w/w) gas-adsorbent charcoal (right).

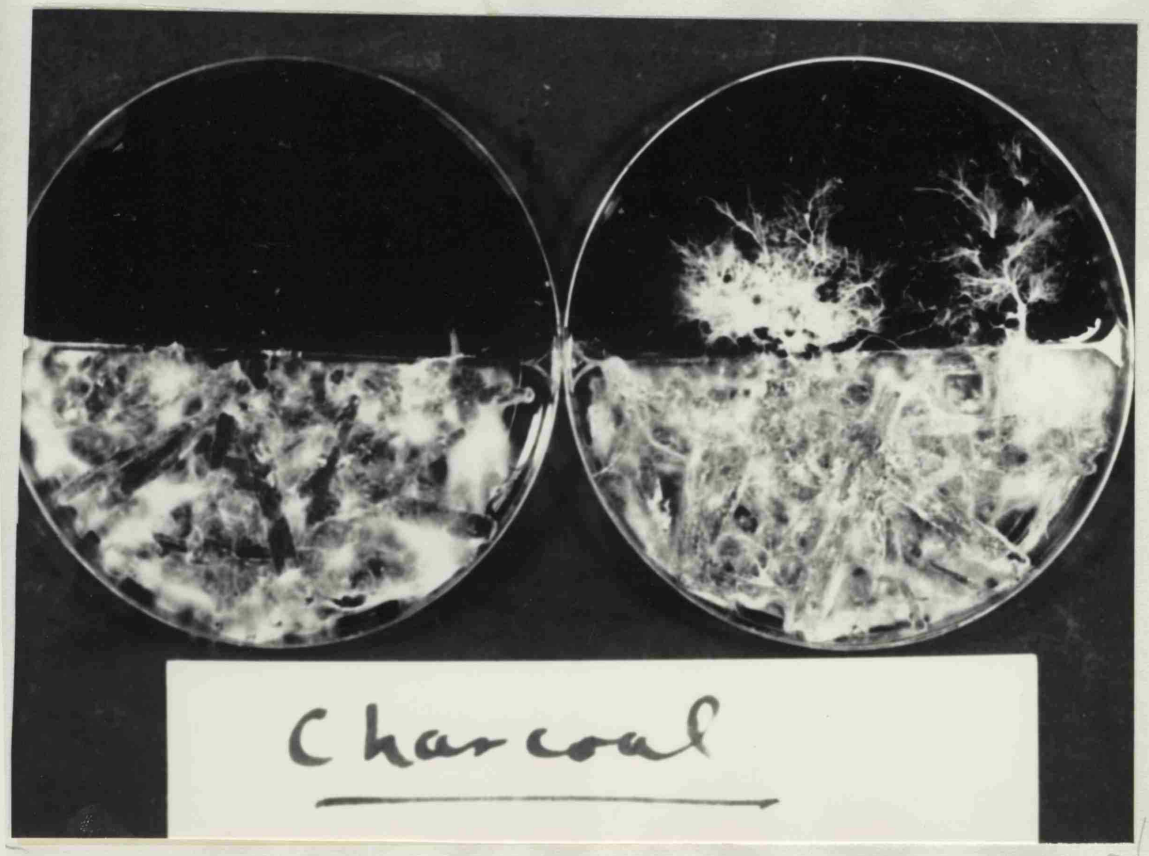

Fig.29. Growth of pure cultures on charcoal alone.
There is virtually no growth in the plate on the left; the plate on the right has initials and strands similar to those in non-sterile casing.

the effect was not repeated with 25% (dry weight) mixtures of Fuller's earth (B.D.H.), calcium silicate, or magnesium silicate with sphagnum peat. Calcium silicate induced stroma-like formations. A more extensive range of substances was examined by placing 0.5 g. of test materials on either side of the same quantity of charcoal on sterile casing in half-plates. Adsorbents and related components thus examined included polyvinyl pyrolidone, (M.W. 10,000; 40,000 and 360,000); anion and cation resins (Dowex 1-18 and 50-W); aluminium, calcium and magnesium silicate; magnesium trisilicate; silica gels (B.D.H. and Merck) and Fullers' earth. None were similar in effect to charcoal but calcium and magnesium silicate inhibited strand growth. Charcoal was consistent in its inhibitory effects, particularly in the first 14 days. Later it became overgrown in some plates where the lids had become inadvertently sealed. Sporophores had formed on the charcoal in 27% of the plates at 28 days after addition of the test materials.

Summary

The role of micro-organisms in sporophore initiation in A. bisporus, first reported by Eger (1961) and confirmed in this study, was not replaced by addition of any of a wide variety of possible microbial metabolites or components, including growth factors and lipids. Some of the latter category may have a stimulatory effect on initiation in non-sterile culture. It was not possible to demonstrate a specific initiating role for sterols in carpogenesis. The chelating agent ethane-diamine-tetra-acetic acid increased the numbers of initials in non-sterile culture but was without effect in sterile conditions.

Further evidence was obtained for the suppression of initiating by supplementing casing with readily available nutrient materials but it was not possible to produce fruit bodies by exposing hyphal strands to complete nutritional deficiency.

Dr. Eger's (1961) observation on the efficacy of activated charcoal in inducing sporophore production by A. bisporus in sterile culture was repeated, indicating that the microflora might act through removal of some metabolites from around the hyphae.

D I S C U S S I O N

A CO_2 requirement has not been previously reported for any stage in the life-cycle of A. bisporus, although Flegg (1952) noted a lowered mycelial yeild in flask cultures supplied with CO_2 free air. The present study demonstrated an absolute CO_2 requirement for hyphal growth in compost and in casing materials; it was also essential for initiation and early development of mushroom primordia.

Observations by Tschierpe (1959) on mycelial cultures in liquid media appear to contradict these findings. Growth was optimal with pure oxygen/nitrogen mixtures, and was progressively inhibited above 4% CO_2. Tschierpe's findings may be attributable to inadequate flushing of metabolic CO_2 from the flasks.

Carbon dioxide clearly plays an important role in sporophore physiology. Initiation was controlled, though not exclusively, by optimal CO_2 levels in casing rather than by a concentration gradient of the gas (c.f. Tschierpe, 1959). The principal effect of increasing CO_2 levels above the optimum for initiation was the maintenance of the vegetative phase of growth, with a concommitant reduction in formation of initials. This observation confirms the findings of Tschierpe (1959) and Tschierpe and Sinden (1964) and probably explains the elongated stipes observed with high CO_2 tensions both experimentally and in growing houses (Lambert, 1933; Tschierpe, 1959). Magnus (1906) in regeneration experiments, demonstrated that pileus tissues were more differentiated than those of the stipe: hence gross elongation of the latter could represent a partial reversion to vegetative growth.

The controlling effect of CO_2 on mycelial growth and sporophore development is surprising in an organism capable of translocation (Lambert, 1962; Nielson and Rasmussen, 1962). No data on the rate of

movement of organic materials through mushroom mycelium is available but Nielson and Rasmussen (1962) recorded translocation of ^{32}P through an 180 cm column of spawn run compost at a rate of approximately 4 cm per day. If applicable to carbon compounds this rate might be insufficient for translocation of readily metabolised products to growing hyphal tips developing into nutritionally deficient casing materials, but this can hardly explain the effect of CO_2 on growth in compost. The response to CO_2 is more readily interpreted if the locus of this control is at the apices of growing hyphae and is independent of translocated materials. Studies by Robertson (1959, 1965) and Park and Robinson (1966) have demonstrated the morphogenetic importance of this region in fungi and its sensitivity to environmental controls.

Carbon dioxide influences development in other fungi. Ingold and Nawaz (1967) reported that fruiting in the gasteromycete Sphaerobolus required CO_2 but was suppressed by increasing levels to 1% or 5%. Accumulation of CO_2 also caused suppression or malformation of sporophores in Collybia velutipes (Plunkett, 1956; Long, 1966) and Schizophyllum commune (Raper and Krongelb, 1958; Niederpruem, 1963). An absolute requirement has been reported for various stages in life cycles of fungi including spore germination, perithecial formation and probably vegetative growth in Chaetomium globosum (Buston, Moss and Tyrrell, 1966); spore discharge in Sordaria fimicola (Ingold and Marshall, 1964); trap formation in Arthrobotrys conoides (Eten and Pramer, 1964) and growth of Mycor and Saccharomyces (Rockwell and Highberger, 1927). It is probable that further investigations would demonstrate that many fungi are influenced more than previously

suspected, by external CO_2 tensions.

Fixation of CO_2 has been studied in sporophore of A. bisporus by Le Roux (1962, 1965, 1968) and by Rast and co-workers (Rast and Bachoffen, 1965, 1967a, b; Bolliger, Rast and Bachoffen, 1966; Wehrli and Rast, 1967; Bachoffen and Rast, 1968). Postulated CO_2 acceptorsincluded pyruvate, phospho-enol pyruvate, aceton and β methyl-crotonyl Co-A., while Wasternack and Reinbothe (1967), studying nitrogen metabolism, have reported incorporation into carbamyl phosphate. Radio-active trace studies have revealed that exogenous CO_2 can enter into the synthesis of Krebs' cycle intermediates, amino-acids, lipids and, via gluconeogenesis, polysaccharides. Wehrli and Rast (1967) found that the most rapid turnover of $^{14}CO_2$ was in the lipid pool, but were unable to correlate any changes in incorporation patterns stages in development of fruit bodies. Le Roux (1968) studying fixation in tissues of sporophores malformed by exposure to relatively high levels of CO_2, reported marked increases in some Krebs' cycle acids, particularly fumarate, citrate and succinate and in the amino-acids glycine, leucine and iso-leucine. Under high (4%) CO_2, uptake of uniformly labelled glucose was reduced but acetate incorporation was increased. Thus the gas may be important as a carbon source for synthetic reactions involving a range of metabolites, particularly when present in high concentrations. Evidence obtained in studies on CO_2 requiring mutants of Neurospora (Charles, 1964; Charles and Broadbent, 1964; Broadbent and Charles, 1965) and Escherischia coli (Charles and Roberts, 1968) emphasises this specialised role of CO_2 in microbial metabolism, particularly with regard

to amino-acid synthesis. One E. coli mutant demonstrated an absolute, irreplacable, CO_2 demand.

The nature of the CO_2 linked reaction in morphogenesis of A. bisporus is not known, but Rast and Bachoffen (1967a) suggest that CO_2 functions in biotin-linked enzyme reactions i.e. synthesis of β-methyl-crotonyl CoA and lipid metabolism. This may be correlated with reports of lipid stimulation of initiation (Schisler and Sinden, 1966; Schisler, 1967). In other fungi the mechanism of CO_2 control of morphogenesis has been most fully studied in Blastocladiella emersoni by Cantino and his associates (reviewed by Cantino, 1966). Resistant sporangieal plants are induced by increased bicarbonate in the medium. The exact control is not known but key reactions appear to be i) reversal of the isocitrate dehydrogenase reaction, resulting in reductive carboxylation of α-keto-glutarate, and ii) increased synthesis of the enzyme isocitratase leading to induction of a new metabolic pathway. These reactions have not been investigated in A. bisporus where the tricarboxylic acid cycle appears to function normally at this point (Rast, 1961; Le Roux, 1966). A closer parallel may exist with CO_2 induction of the yeast form in Mucor rouxii (Bartnicki-Garcia and Nickerson, 1962a). In isotope experiments the bulk of the labelled CO_2 appeared in aspartate which might contribute to formation of a mannan-protein complex in yeast cell walls (Bartnicki-Garcia and Nickerson, 1962b). This interpretation could apply to A. bisporus since Le Roux (1962) found that aspartate was the most heavily labelled metabolite in sporophore slices exposed to $^{14}CO_2$.

Although CO_2 clearly exerts an over-riding morphogenetic control

in non-sterile condition other factors are involved. Within the range 104 to 1000 p.p.m. CO_2, initiation was associated with a marked check in mycelial growth which occurred about 7 days after casing. This retardation of strand growth preceded the first appearance of clearly formed initials by 24 to 48 hours on many occasions, corroborating Eger's (1961) observations with the "Halbschalentest".

The studies by Eger (1960 to 1965b); O'Donoghue (1962) and Thomas et al (1964) have emphasesed the participation of the casing microflora. These conclusions are supported in the present investigation by the consistent failure to obtain primordia in the absence of a viable casing microflora. There can be little doubt that initiation in non-sterile or inoculated casing reflects the activity of micro-organisms in the vicinity of mushroom hyphae. In view of the wide variety of casing materials which have been successfully employed in this and other investigations (Schisler, 1957; Eger, 1961) i.e. soil, peat, sand, gravel, coke and pummice; it is unlikely that one specific organism induces sporophore initiation. The repeated failure to promote mushroom formation with single isolates suggests that a balanced population involving a variety of organisms in equilibrium is reponsible. However, Schisler (1957), Koch (1958), Lockard (1962) and Lockard and Kneebone (1962) have claimed production of sporophores in aseptic culture. Both Schisler and Lockard employed the same type of apparatus i.e. aerated Erlenmeyer flasks with autoclaved compost and soil casing. Filtration of the incoming air was effected by means of cotton-wool packed small bore inlet tubes on these flasks; a method found inefficient both in this study and by Eger (1961). In Koch's

experiments the horse manure and the loess soil casing were autoclaved once, for thirty minutes, a procedure which may be insufficient to ensure sterility. Thus, there is an element of uncertainty concerning both these reports.

In Eger's view (1961-1965b) the active microflora is closely associated with mycelial strands in the casing layer. Direct observation of strands in the present study demonstrated the localised presence of bacteria adjacent to the hyphae and growing parallel to the surfaces; greater numbers were associated with moribund or decaying tissues. Analogous situations have been described by a number of workers (c.f. Lockwood, 1967). In such an intimate environment as the hyphosphere removal of metabolites from the hyphae and their vicinity is likely to occur and could explain the effects of gas adsorbent charcoal on A. bisporus first noted by Eger (1961) and confirmed in this study.

Gas-adsorbent charcoal proved to be the only material capable of inducing sporophore formation in sterile culture. It was active alone or in various mixtures with buffered sphagnum peat. Sporophore formation appeared normal in its presence, and the development of hyphal strands closely resembled growth over unsterilised casing. It appears that the effect of charcoal can be overcome by accumulated CO_2, as it was overgrown by strands in petri-dishes accidentally sealed by condensation around the rim. Gas adsorbent charcoal has affinity for a wide range of polar substances and it proved impossible to define the metabolites involved by use of more selective adsorbents.

Many reports of interaction between bacteria and fungi leading to development of reproductive structures in fungi (Hawker, 1950;

Dade, 1960) appear to relate to supply of some essential growth factor. This might be termed a "synergistic" effect. A clear example is fruiting by thiamine requiring mutants of Schizophyllum commune in the presence of Bacillus mycoides (Raper and Krongelb, 1958). Similar considerations may apply to observations of bacterial stimulation of oospore formation in Phytophthora cinnamomi (Marx and Haasis, 1965; Chee and Newhook, 1966) and to the stimulatory effect of Bacillus psilocybe in fruiting in a number of agarics including A. bisporus (Urayama, 1960, 1961). However, this does not appear to be the mechanism for microbial involvement in mushroom sporophore initiation since added vitamins had no effect.

A closer analogy exists between sporophore initiation and the phenomenon of soil mycostasis first described by Dobbs and Hinson (1953) and reviewed by Lockwood (1964) and Jackson (1965). Many fungal spores fail to germinate in natural soils but do so in soils pretreated by autoclaving, washing with organic solvents, or addition of nutrients such as glucose. Attempts to isolate specific organisms or active materials involved in mycostasis have proved inconclusive. Recent work suggests that removal of nutrients from spore surfaces may be important. Ko and Lockwood (1967) noted that spores susceptible to mycostasis required an external energy source for germination and that mycostasis could be simulated by prolonged leaching of spores with sterile distilled water. Similar findings were recorded by Adams, Lewis and Papavizas (1968) working with Fusarium solani f. phaseoli. Further corroboration for this mechanism derives from Cooke's (1967) observation that recently exposed glacial soils were strongly fungistatic.

The soil microflora that associates with fungal structures can thus function as a "nutrient sink". This might involve creating a simple nutritional deficiency in soil adjacent to spores or the active removal of their metabolites. Such a property of natural soils may constitute a reproductive control mechanisms for soil saprophytic fungi. Thus, perfect forms of Rhizoctonia spp. were produced by employing a casing technique (Stretton, McKenzie, Baker and Flentje, 1964).

Phosphate, di-sodium ethane diamine tetra-acetic acid (EDTA) and some lipid sources showed stimulatory activity in non-sterile culture but were without effect under sterile conditions, indicating that they do not participate in sporophore induction. These substances may have primarily affected the casing microflora rather than acting directly upon the mushroom mycelium. Phosphate accumulation as polyphosphate is a characteristic of mushroom sporophore development (Kulav, Kritskii and Belozerskii, 1960; Thielle, 1968), but Treschow (1944) recorded that concentrations above 0.007 M were inhibitory to mycelial growth in a defined medium. This toxicity might account for the limited strand growth in the presence of phosphate buffer. The activity of E.D.T.A. is difficult to interpret. Chelation of calcium could constitute a possible mechanism as the ion is required for mycelial growth (Treschow, 1944) and contributes to formation of calcium oxalte crystals in hyphal surfaces (Eger and Sücker, 1964). Due to use of limestone as a buffer, the ion would be present in excess, however, and chelation of other metals is more likely.

The stimulatory effects of lipids, possibly steroids, reported by Schisler and Sinden (1966), Schisler (1967) may not represent a

specific role in sporophore initiation but an increased requirement at this stage due to proliferation of hyphal membranes in which they are important components. Hughes (1962) recorded a variety of glycerides in mushroom sporophores, with a relatively large amount of free sterol. The principal fatty acid of the ten identified by this worker was linoleic acid, 65% to 75% of the total. Failure to demonstrate a link between steroid function and polyene inhibition in A. bisporus may represent inability to incorporate steroid in the form supplied rather than an alternative action mechanism for polyenes to that proposed by Gottliebetal (1961). As, the activity of polyene antibiotics applied to vegetative and reproductive phases of growth, this suggests that steroid is important in both phases of mushroom development.

The present findings militate against participation of any volatile factors (Mader, 1943; Stoller, 1945, 1952; Schisler, 1957) produced in compost, apart from CO_2, in fruit body formation. Sporophores consistently formed in the casing of non-sterile growth chambers supplied with defined air mixtures of suitable CO_2 content. Under these conditions volatile metabolites produced within the compost were excluded from the distal portions of strands where initials formed. There could be no parallel with the situation envisaged by Schisler (1957) who postulated trapping of a volatile "hormone" within the casing. The negative results obtained with ethylene, a metabolite of the mushroom (Lockard, 1962; Lockard and Kneebone, 1962), might be criticised on the grounds that the concentration range examined was too restricted, although the gas has been reported biologically active at a dilution of 5×10^{-8} (Biale, 1960).

The results of the present investigation indicate that the essential functions of the casing layer in sporophore initiation relate to: -

1) A retardation in hyphal growth brought about through the activity of the hyposphere microflora; operating most effectively between 340 and 1000 p.p.m. CO_2.
2) An over-riding control of the process by CO_2, which when concentrations are increased overcomes the microbial effect.

Mycelial strands of A. bisporus are structurally differentiated into lead and tendril hyphae (Matthew, 1961) and it is uncertain whether both types are involved in morphogenesis. It can be argued that both these hyphae when penetrating the casing layer are subjected to a complex interaction between microbial and CO_2 controls. There also appears to be link with the nutrient status of the substratum in their immediate vicinity. These questions are best considered in relation to the markedly differing responses to CO_2 that occurred in sterile or non-sterile peat casing and in non-sterile compost.

Within sterile casing vegetative growth reached a maximum at approximately the normal atmospheric level of CO_2. By contrast, sporophore production in non-sterile casing could occur at the same concentrations. In non-sterile compost the situation was in many respects analogous to that in sterile casing, and no sporophores were produced. It could be postulated that the micro-organisms are inhibiting the hyphal response to CO_2 in non-sterile casing, during growth from a nutrient base, but are ineffective in the presence of an adequate nutrient source.

A number of alternative mechanisms could account for interference

with hyphal responses to CO_2 in non-sterile casing. Some of these, action of other volatile agents such as ethylene, and absence of nutrients have already been discussed in other contexts and appear unlikely to be involved. Volatiles, of the staling factor type (Park, 1963, 1964) produced by the soil microflora rather than the mushroom mycelium would probably not be active in the continuously ventilated casing of the culture chambers. The nutrient status of autoclaved, washed casing was similar to that of untreated peat, but growth although lagging slightly behind that in unwashed sterilised casing, continued unchecked and no sporophores were formed. Interference by antibiotics, in the restricted sense of activity by potent, complex antimetabolites would not account for the hyphal responses to increasing CO_2. Such substances induce gross structural and biochemical changes unlikely to be counteracted by increased supply of a simple compound. Antifungal antibiotics such as the polyene group and griseofulvin checked mushroom mycelial growth but did not induce sporophore formation. Further evidence against antibiotic action was the ineffectiveness of culture filtrates and (Eger, 1961) bacteriologically filtered soil extracts. Furthermore it is difficult to reconcile antibiotic action with the results of employing charcoal as a casing material.

Other possible mechanism include, competition for CO_2 supplied to the system· interference with uptake of the gas by the hyphae, and removal of materials: possibly those in which CO_2 is fixed, from the mycelium. The first was extremely unlikely in the rapidly aerated growth chambers. Interference with uptake is also difficult to conceive. According to Loomis (1959) carbon dioxide gas readily

diffuses through cell membranes, and there is no evidence among fungi for active uptake via carbonic anhydrase or similar systems. This latter possibility was examined by adding sulphanilamide, an inhibitor of carbonic anhydrase to sterile cultures, without effect. Removal of hyphal metabolites appears to be in accord with experimental observations to date, and agrees with the concept that the hyphosphere flora acts as a nutrient "sink", as suggested by the findings with charcoal. Increased CO_2 could overcome the microbial effect by inducing further synthesis of the relevant metabolites.

The reports on CO_2 fixation mechanisms in A. bisporus, discussed earlier, revealed that CO_2 could be involved in the synthesis of Krebs' cycle acids and their derivatives, amino-acids, lipids and polysaccharides. Short chain aliphatic acids are likely to be present in the hyphosphere, being commonly secreted into growth media during glycolysis in fungi. Of these, oxalic acid appears to be a normal end-product of metabolism in agarics when available carbon cannot be used for growth (Cochrane, 1958). This substance is produced copiously by the cultivated mushroom (Tsao, 1963; Eger and Sucker, 1964), and could be derived from oxalo-acetate, the first product of CO_2 fixation via phospho-enol pyruvate (Bachoffen and Rast, 1968). It is tempting to postulate that this is the substance removed by the micro-organisms. An objection to this view is the report by Eger and Sucker (1964) that oxalate, as the calcium salt, is produced by mushroom hyphae and initials when active growth is retarded. This is important as the metabolites sequestered by the hyphosphere flora must be produced during normal hyphal extension. Tschierpe and Sinden

(1965), however, reported oxalic acid as one of the aerobic products of mushroom mycelium, and it is possible that small quantities, insufficient for visible calcium oxalate formation are produced continuously by hyphal tips during vegetative growth. Citric acid is also a product of CO_2 fixation in sporophore tissues (Le Roux, 1968), and apart from its key role in the tricarboxylic acid cycle is of interest through its control of fatty acid synthesis in animal tissues (Lynen, Matsuhashi, Numa and Schweizer, 1963).

Removal of metabolites from the vicinity of the hyphae could induce morphogenesis by altering the plasticity or permeability of the apices through changes in the external environment or operate a feed-back mechanism by lowering internal metabolite concentrations. The latter event could bring into operation the genetic sequences controlling carpogenesis. Further, it seems likely from the action of charcoal in sterile culture that the presence round the hyphal tips of continuously excreted metabolites is a prerequisite for undifferentiated vegetative growth. Production of these substances would require an adequate carbon source, which in the case of A. bisporus strands invading casing can either be derived from reserves in the compost mycelium or via CO_2 fixation.

An interesting aspect of this nutritional "sink" hypothesis is that it could play a role in the increased flow of nutrients towards developing sporophores. Although Wessels (1965) found that the first stages of initiation in thiamine induced carpogeneis of Schizophyllum commune occurred before detectable changes in medium nutrients, one of the key processes in subsequent development was mobilisation of reserve glucan in the vegetative mycelium and its translocation to the primordia.

Similarly Madelin (1956) produced evidence that further growth of individual primordia in Coprinus lagopus depended on intensification of a 'nutritional flow towards them. In both these examples, however, it should be noted that initiation had been induced before the appearance of enhanced translocation.

This hypothesis on control of sporophore initiation in A. bisporus presents carpogenesis as a response to irreplacable removal of metabolites from hyphal apices as they grow from a nutrient base, through the agency of the casing microflora. Increased nutritional status, including enhanced CO_2 supply, permits further synthesis of the lost metabolites and normal hyphal strand extension. The localised nature of sporophore induction must be stressed. An objection to this view is that increasing CO_2 might counteract microbial inhibition of hyphal growth by reducing bacterial numbers rather than through direct effects on mycelial metabolism. Such an explanation does not, however, apply to growth over charcoal which is probably also enhanced by higher CO_2 levels. If fruiting is controlled by the active removal of metabolites by micro-organisms the hypothesis is in broad agreement with Klebs' (1900) views on fungal reproduction but it does not support the Klebsian inter retation of Lambert (1933) and Edwards (1949) who visualised the casing as a starvation zone.

Any hypothesis on the mechanism of fruit body formation must, ultimately, be interpretable at the molecular level. A hindrance to the understanding of carpogenesis in the mushroom is the lack of information on the nature and relative importance of metabolic routes in the vegetative and reproductive phases. Some indications of differences in metabolism derive from Tschierpe and Sinden's (1965)

gas chromatography study which indicated a greater diversity of fermentation products from the mycelium and supported Le Roux's (1962, 1963) suggestion that the hexose monophosphate shunt might be an important glycolytic path in sporophores. According to Kulaev et al (1960) polyphosphates accumulate in sporophore tissues in association with spore production. Recently Eger (1968) has provided some evidence for the presence of internal initiation inducers in fruit bodies of A. bisporus and Pleurotus florida. Low molecular weight substances from both fungi brought about primordia formation when applied to P. florida mycelium. It is evident from these few observations that empirically tested substances promoting initiation may not be involved in the inducing reaction itself but could represent intermediates in later reaction sequences. In interpreting results from experiments with unsterilised materials it is impossible to distinguish between substances acting directly upon the mycelium and those exerting their effects primarily on the microflora, unless similar reactions also occur in sterilised substrates.

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